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TRANSLATING PROTEOMICS INTO ENVIRONMENTAL HEALTH:

Biomarkers discovery for tobacco smoke-induced biological damage

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Resumo

O fumo do tabaco é a principal causa de morte evitável em todo o mundo, e resulta em avultados custos na saúde e produtividade. Desde 1964 que mais de dois milhões de não-fumadores morreram, por causas relacionadas com a exposição involuntária ao fumo do tabaco.

A combustão de tabaco em espaços fechados, origina o chamado fumo de tabaco ambiental (FTA), que se dissemina e acumula por todo o espaço. Estima-se que 50% das crianças, em todo o mundo, estão regularmente expostas ao FTA e que mais de 40% possuem, pelo menos, um progenitor fumador. Como resultado, num só ano, morreram mais de 160 mil crianças.

O FTA é constituído por uma mistura de sete mil substâncias químicas, das quais 70 são cancerígenas. Entre estas incluem-se os hidrocarbonetos aromáticos policíclicos, um grupo de poluentes do ambiente produzido durante a combustão incompleta de matéria orgânica. A exposição a estes químicos aumenta significativamente o risco de desenvolver várias doenças, nomeadamente as principais causas de morte nas últimas décadas, tais como o cancro do pulmão, a doença pulmonar obstrutiva crónica, as infecções do tracto respiratório inferior, a doença cardíaca isquémica e o ataque cardíaco.

A Organização Mundial de Saúde alerta para o facto de não existir um nível de exposição ao FTA isento de risco e que em certos grupos, como os trabalhadores de espaços públicos fechados onde ainda é permitido fumar, esse risco é particularmente elevado.

Em 2008, entrou em vigor em Portugal uma nova legislação (Lei n. 37/2007), que condiciona o acto de fumar em espaços públicos fechados. Assim, os proprietários de espaços de lazer, puderam optar por proibir ou permitir totalmente o acto de fumar no seu interior, mediante o cumprimento de condições determinadas na lei. Outros estabelecimentos, designados mistos, passaram a incluir áreas distintas para fumadores e para não-fumadores.

O presente projecto iniciou-se no ano seguinte à entrada em vigor desta nova legislação, com o objectivo de compreender o seu impacto na saúde de trabalhadores da área da restauração. Para o efeito, foram avaliados restaurantes na área de Lisboa, quanto a

eventual contaminação do ar com FTA, seguida da pesquisa de biomarcadores moleculares de exposição ao FTA, nos trabalhadores destes mesmos espaços.

No total, 25 restaurantes foram incluídos no estudo, 13 mistos, com áreas designadas para fumadores (ADF) e para não-fumadores (ADNF) e 12 restaurantes onde era totalmente proibido fumar. Quatro cantinas públicas foram incluídas, como referência de espaços de não-fumadores, uma vez que nestes recintos é proibido fumar desde 1984. Tendo em conta que o ar interior contacta com o do exterior, também se monitorizaram 16 espaços exteriores, contíguos àqueles restaurantes.

Destes espaços, foram recrutados 97 trabalhadores, dos quais foram obtidas 87 amostras de urina, 81 amostras de sangue e 27 amostras de células do epitélio nasal. Adicionalmente, efetuaram-se exames clínicos e um questionário detalhado relativo ao estilo de vida. Todos os indivíduos com doença respiratória foram excluídos do estudo.

Para a monitorização da eventual contaminação do ar interior com FTA mediram-se, entre outras, as concentrações de partículas finas, de dióxido de carbono e de monóxido de carbono, e estimou-se a concentração de hidrocarbonetos aromáticos policíclicos. Procedeu-se ainda ao levantamento de informação relativa à tipologia do espaço e ventilação.

Os resultados da monitorização do ar revelaram uma elevada contaminação de partículas finas e consequentemente de hidrocarbonetos aromáticos policíclicos, em todas as ADF, relativamente às ADNF e cantinas. Além disso, registou-se uma maior mediana da concentração de monóxido de carbono nas ADF. É de salientar que a concentração urinária de cotinina, um metabolito da nicotina, foi significativamente superior nos trabalhadores das ADF, em relação aos trabalhadores das ADNF e de restaurantes onde era totalmente proibido fumar, independentemente do seu hábito tabágico.

Estes resultados demonstram que a exposição a níveis potencialmente perigosos de FTA, se mantém elevada em restaurantes onde existem ADF. Tal ocorre, independentemente das medidas de protecção implementadas após a referida legislação, nomeadamente dos sistemas de ventilação instalados. Tendo em conta que todos os trabalhadores incluídos no estudo apresentavam parâmetros respiratórios normais, decidiu-se investigar possíveis alterações moleculares induzidas por esta exposição, principalmente em trabalhadores que nunca fumaram.

Não se encontraram diferenças significativas na concentração de marcadores de stress oxidativo, nomeadamente na capacidade antioxidante total do plasma e na 8-hidroxi-2'-desoxiguanosina do soro sanguíneo. Contudo, ao nível do DNA e proteínas, foram encontradas diferenças nos trabalhadores das ADF.

Após a exposição dos leucócitos a um agente mutagénico, um ensaio do cometa revelou que os trabalhadores expostos ao FTA têm um maior capacidade de resposta aguda na reparação do DNA, em relação aos não-expostos.

Em relação ao plasma, as amostras foram enriquecidas de proteínas menos abundantes pela técnica da depleção das mais abundantes. De seguida, foram misturadas em pools e submetidas a electroforese bidimensional diferencial (2D-DIGE). O padrão de separação das proteínas foi analisado pelo software SameSpots e, as proteínas assinaladas como diferencialmente expressas com base na intensidade do spot, foram identificadas por espectrometria de massa, no instrumento MALDI-TOF/TOF.

Esta metodologia identificou nove proteínas diferencialmente expressas nos trabalhadores das ADF. Entre essas, destacam-se a *ceruloplasmin* (CP) e a *inter-alpha-trypsin inhibitor heavy chain 4* (ITIH4), duas proteínas de resposta inflamatória aguda. Este trabalho foi pioneiro na identificação, em simultâneo, de um elevado número de isoformas destas proteínas. Adicionalmente, ambas apresentavam um padrão de expressão comum, isto é, quando se comparam indivíduos expostos ao FTA com os não-expostos, há uma diminuição da concentração das isoformas de elevada massa molecular em relação às de baixa massa molecular.

Para complementar e corroborar estes resultados, procedeu-se a um segundo estudo proteómico em amostras individualizadas de plasma e do epitélio nasal, por cromatografia líquida associada a espectrometria de massa, utilizando o equipamento ESI-LTQ-Orbitrap. Estas experiências geraram uma grande quantidade de dados e, para garantir o seu escrutínio rigoroso, procedeu-se à sua análise no software Progenesis QI. Ao todo, foram identificadas 521 proteínas no plasma e 3384 no epitélio nasal, das quais 38 estavam diferencialmente expressas nos trabalhadores expostos ao FTA, quando comparadas com os trabalhadores não-expostos.

A análise funcional destas proteínas, demonstrou que as mesmas estão envolvidas em vários processos de doenças associadas à exposição ao fumo do tabaco. Assim, decidiu-

se validar um subgrupo de seis proteínas. Destas, cinco foram validadas como possíveis biomarcadores de exposição ao FTA, através da tecnologia de *specific reaction monitoring*, nomeadamente a *gelsolin* (GSN), a *alpha-1-acid glycoprotein* (ORM2), o *elongation factor 2* (EF2) e as proteínas ribossomais *40S S8* (RPS8) e *60S L13* (RPL13).

Com estes estudos, e respectiva dissertação, espera-se contribuir para uma compreensão mais alargada dos mecanismos de resposta à exposição ao FTA. A precedência destes mecanismos moleculares, relativamente aos primeiros sinais clínicos de doença, aponta para a importância das proteínas identificadas, como potenciais biomarcadores para a saúde ocupacional. Após a validação destas proteínas, numa amostra mais alargada de indivíduos, as mesmas poderão vir a ser utilizadas na rotina clínica, como ferramenta de monitorização da exposição ao FTA. Os resultados deste trabalho também alertam para a necessidade de uma revisão urgente da actual legislação, no sentido de garantir a efectiva protecção ocupacional, ou da saúde pública em geral, da exposição involuntária ao fumo do tabaco.

Palavras-chave: Fumo do tabaco, saúde ocupacional e biomarcadores

Abstract

Tobacco smoke is the number one preventive cause of death and causes massive losses in health and productivity worldwide, each year. Since 1964, near two million non-smokers died from involuntary exposure to tobacco smoke.

When people burn tobacco products, secondhand smoke (SHS) spreads, fills and accumulates in enclosed spaces. Nearly 50% of children regularly breathe air polluted by tobacco smoke in public places and over 40% have at least one smoking parent. As a result, in a single year more than 160 thousand children have died from SHS related illness.

Tobacco smoke contains more than seven thousand chemicals, including 70 carcinogens. Polyaromatic hydrocarbons, a group of environmental pollutants produced during incomplete combustion of organic materials, are among those. Exposure to these chemicals significantly increase the risk for several diseases, namely ischemic heart disease, stroke, lower respiratory infections, lung cancer and chronic obstructive pulmonary disease. All are top major killers of the last decades.

According with the World Health Organization, there is no safe level for SHS exposure and some groups, such as the personnel working in leisure venues where smoking indoors is allowed, are particularly at risk.

In 2008, smoking in public places was partially banned, under the implementation of a new legislation in Portugal (Law 37/2007). Accordingly, leisure venues owners could choose if it would be entirely allowed or prohibited to smoke indoors. Other venues became a mixture including both designated areas for smokers and for non-smokers inside their premises. A year after, the will to understand the health impact of this new legislation, in these venues workers, driven this project. For that purpose the indoor air of restaurants in Lisbon was evaluated for tobacco smoke contaminants, followed by the search for biomarkers of SHS exposure, in these venues workers.

In total 25 restaurants were included in the study, where 13 were mixed with both smoking and non-smoking designated areas, SDA and NSDA, and 12 were entirely smoke-free. Four canteens were also included as a reference of smoke-free places, considering that they have been free from SHS since 1984. Furthermore, the fact that outdoor contaminants easily penetrate indoors, 16 of the 25 restaurants were monitored outdoors as well.

A total of 97 employees were recruited. In summary 87 provided a urine sample, 81 provided blood and 27 provided cells from nasal epithelia. Additionally workers filled a detailed lifestyle survey and went through clinical evaluation. All subjects with respiratory disease were excluded from the study.

To evaluate possible SHS-indoor air contamination, fine particles, carbon dioxide and carbon monoxide concentrations were monitored and polyaromatic hydrocarbon levels were estimated. Information regarding typology and ventilation was also recorded.

The results from indoor air monitoring demonstrated high contamination with fine particles in all restaurants with SDA, in comparison with NSDA or canteens. Moreover, carbon monoxide median level was higher in SDA. Importantly, the urinary concentration of cotinine, a metabolite of nicotine, was increased in workers from SDA, compared with NSDA and entirely smoke-free restaurants, regardless of their smoking status.

All together, these results demonstrated that exposure to hazardous SHS levels remain high in restaurants where smoking is still allowed, regardless of the protective measures adopted. Moreover, it was proved that workers in SDA are still effectively exposed to SHS, inhaling tobacco combustion sub-products.

The fact that these workers are clinically healthy, motivated the search for molecular changes that could be induced by occupational SHS exposure, mostly in subjects that never smoked. After monitoring two oxidative markers, namely the plasma total antioxidant status and serum 8-hydroxy-2-deoxy-guanosine, we found no significant differences between SHS-exposed and unexposed workers, conversely to the analysis of DNA and proteins.

After challenging leucocytes with a mutagenic agent, a comet assay revealed that SHS exposed workers had a higher acute DNA repairing response.

The plasma samples were enriched with the medium and lower abundant proteins, by the immunodepletion of the higher abundant proteins. Then the samples were pooled, and run by two dimensional difference in gel electrophoresis (2D-DIGE). The pattern with the separated protein spots, in the gel image, was analyzed with SameSpots software and those indicated to be significantly differentiated run in a tandem mass spectrometry approach in, a MALDI - TOF/TOF. This approach identified nine proteins that significantly differentiated the workers that were exposed to SHS. Out of these, two acute-phase inflammation proteins, ceruloplasmin (CP) and inter-alpha-trypsin inhibitor heavy chain 4 (ITIH4), became prominent.

For the first time, to our knowledge, both were represented with a high number of isoforms. In addition, they exhibited a common expression pattern, where all high-molecular-weight isoforms suffered a decrease in their concentration, while low-molecular-weight isoforms suffered an increase, in SHS exposed workers.

In order to complement and corroborate these results, a second proteomics approach was performed in individual samples of plasma and nasal epithelia, by liquid chromatography coupled with a tandem mass spectrometry instrument, a ESI-LTQ-Orbitrap. These experiments generated huge amounts of data that was submitted to Progenesis QI software, for a proper examination. In total 521 proteins were identified in depleted plasma samples and other 3384, in nasal epithelia. All in all, 38 proteins were significantly different in SHS-exposed workers, compared with the unexposed.

Functional analysis revealed that these proteins are involved in various processes already proved to be induced by exposure to tobacco smoke. Therefore a subset of six proteins, was submitted to an additional validation step. Aided by a targeted specific reaction monitoring technique we were able to validate five, as candidate biomarkers namely gelsolin (GSN), alpha-1-acid glycoprotein (ORM2), elongation factor 2 (EF2) and ribosomal 40S S8 (RPS8) and 60S L13 (RPL13) proteins.

With this work and dissertation, it is expected to widen the knowledge of the SHS-induced molecular mechanisms. The fact that these molecular mechanisms precede the first clinical symptoms of disease, makes their routine use in health checkups critical, towards the protection of occupational health.

Furthermore, to fully protect workers, or public health in general, from SHS exposure, a law revision is urgently advised in Portugal.

Key-words: Tobacco smoke, occupational health and biomarkers.

Symbols and Abbreviations

1DE	One dimensional electrophoresis
2DE	Two-dimensional electrophoresis
8-OHdG	8-Hydroxydeoxyguanosine
ABTS	2,2'-azino-bis-(3-ethylbenzthiazoline sulphonate)
ACN	Acetonitrile
AGP	Alpha-1-acid glycoprotein
AmBic	Ammonium bicarbonate
ANOVA	Analysis of variance
AP2A1	Isoform A of AP-2 complex subunit alpha-1
ARCN1	Archain 1
ASS	Argininosuccinate synthetase
ATS	American Thoracic Society
B[a]P	Benzo[a]pyrene
BALF	Bronchoalveolar lavage fluid
BMI	Body mass index
Cant	Canteens
CC	Correlation Coefficient
CFHR2	Isoform long of complement factor H-related protein 2
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulphonate
CID	Collision-induced dissociation
CO	Carbon monoxide
CO_2	Carbon dioxide
COPA	Isoform 1 of coatomer subunit alpha
COPD	Chronic obstructive pulmonary disease
CP	Ceruloplasmin
Da	Dalton
DALYs	Disability-adjusted life years
DDM	Data dependent mode
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
EF2	Elongation factor 2
ELISA	Enzyme-linked immunosorbent assay
ER	European region
ERS	European Respiratory Society
ESI	Electrospray Ionization
ETHE1	Persulfide dioxygenase ETHE1
ETS	Environmental tobacco smoke
f	Fold change

F11	Coagulation factor XI
F5	Coagulation factor V
FA	Formic acid
FCTC	Framework convention on tobacco control
FDR	False discovery rate
FEV1	Forced expiratory volume in one second
FT	Fourier transformed
FVC	Forced vital capacity
FWHM	Full width at half maximum
GNB2L1	Guanine nucleotide-binding protein subunit beta-2-like 1
GOLD	Global Initiative for obstructive lung diseases
GSN	Gelsolin
HCl	Hydrochloride
HMWI	High-molecular-weight isoforms
HNRPAB	Heterogeneous nuclear ribonucleoprotein A/B
HPLC	High-performance liquid chromatography
HSD17B10	Isoform 1 of 3-hydroxyacyl-CoA dehydrogenase type-2
IAM	Iodoacetamide
IAQ	Indoor air quality
Ig	Immunoglobulin
IPG	Immobilized pH gradient
IS	Internal standard
ITIH	Inter-α-trypsin inhibitor heavy chain
KTN1	Isoform 1 of kinectin
LC	Liquid chromatography
LMWI	Low-molecular-weight isoforms
LTQ	Linear ion trap
LY6D	Lymphocyte antigen 6D
MALDI	Matrix-assisted laser desorption/ionization
MAOB	Amine oxidase [flavin-containing] B
MARS	Multiple affinity removal system
Max	Maximum
Min	Minimum
mob	Mobile phase
MS	Mass spectrometry
MSN	Moesin
MWCO	Molecular weight cut-offs
m/v	Mass volume fraction
NaCl	Sodium chloride
NEC	Nasal epithelial cells
NS	Non-smokers

NSDA	Non-smoking designated areas
NSE	Non-smokers exposed to SHS
NSNE	Non-smokers not exposed to SHS
Nsro	Non-smoking rooms
O	Outdoor
OR	Odds ratios
ORM	Alpha-1-acid glycoprotein
p	p value
ppm	Parts per million
PAGE	Polyacrylamide gel electrophoresis
PAHs	Polyaromatic hydrocarbons
PBS	Phosphate buffer saline
PI	Protease inhibitor
PIKE	Protein information knowledge extractor
PLCH	Pulmonary Langerhans cell histiocytosis
$PM_{2.5}$	Fine particles
PPAHs	Particulate phase of SHS
QL	Quantification limit
RBC	Red blood cell
ROS	Reactive oxygen species
RPL13	60S ribosomal protein L13
RPL14	60S ribosomal protein L14
RPS8	40S ribosomal protein S8
RPS11	40S ribosomal protein S11
RSP	Respirable/fine particles
RT	Room temperature
S	Smokers
SCX	Strong cation exchange
SD	Standard deviation
SDA	Smoking designated areas
SDS	Sodium dodecyl sulphate
SE	Smoker exposed
EMS	Ethyl methane sulfonate
SERPINA5	Plasma serine protease inhibitor
SFre	Entirely smoke-free restaurants
SHS	Secondhand smoke
SNE	Smokers not exposed to SHS
Sre	Restaurant entirely designated for smokers
SRM	Specific reaction monitoring
Sro	Smoking rooms
SYNE1	Nesprin-1
SYNPR	Synaptoporin

TAS	Total antioxidant status
TFA	Trifluoroacetic acid
TOF/TOF	Time-of-flight
Tris	Tris(hydroxymethyl)aminomethane
TSQ	Triple stage quadrupole
TXN	Thioredoxin
US	United States of America
USO1	Isoform 1 of general vesicular transport factor p115
v	Version
v/v	Volume fraction
VCL	Isoform 2 of vinculin
WAS	Wiskott-Aldrich syndrome protein
WDR81	Isoform 1 of WD repeat-containing protein 81
WHO	World Health Organization
α	Significance level
α-CHCA	α-Cyano-4-hydroxycinnamic acid

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Supplementary information:

Preface

For those who have followed my education, it was not surprising that my PhD was dedicated to environmental health. In fact, promoting public health has driven all my life, or at least, as far as I can remember.

In 2008, the leisure industry went through several changes under the specification in the newly implemented tobacco law 37/2007. These changes were expected to protect workers, from involuntary exposure to tobacco smoke, also called Secondhand smoke (SHS). By 2009, the lack of the outcome information prompted the start of this project. During the next four years, a series of studies were performed in order to understand if these venues workers were effectively protected from SHS. Additionally, supported by proteomics, our group aimed to fill the gap knowledge in the molecular mechanisms induced by SHS exposure. Unraveling these mechanisms is crucial, since they lead non-smoker workers to chronic tobacco related diseases, such as lung cancer, heart disease, stroke and even death.

In this dissertation, we will travel from a macro environmental perspective towards the micro molecular world of proteins.

The brief introductory chapter describes the so called tobacco epidemic, provides a context for SHS exposure worldwide and at the national level. It also includes a short explanation of proteomic studies and their application in biomedical research.

The four chapters that follow have the same structure and present the main studies of this PhD. The first two studies were published in 2012 and 2013 respectively, and the last two studies are under submission process. Each chapter begins with an introduction, to contextualize the study, followed by the material and methods applied, in order to achieve the results, which are discussed and dissected in main conclusions.

The first study reveals the indoor air quality analysis, as well as the evaluation of SHS acute exposure of these venues workers. The second study discloses the preliminary results at both DNA and protein level. The third and fourth studies, describe the proteomic experiments, where plasma and nasal epithelial cells were analyzed in parallel, to assess molecular findings at both systemic and local airway level.

Finally, all results are wrapped in concluding remarks followed by future perspectives.

Introduction

According with the World Health Organization (WHO), tobacco is the world leading cause of preventable morbidity, mortality and incapacity, and caused more than half trillion dollars of economic damage (WHO, 2013).

The European Region (ER) has one of the highest proportions of deaths in adults over 30 (16% of all deaths), in contrast with the 12% global average. Every year, tobacco smoke kills nearly six million people in the world, seven thousand in the ER (WHO, 2012c).

It is estimated that around one third of the world population aged 15 or over, smokes and that one third of adults and 40% of children, are involuntarily exposed to their smoke. Exposure to other people's smoke, also called, secondhand smoke (SHS) causes more than 600 thousand of all year deaths, nearly 1% of worldwide mortality (WHO, 2014b). These deaths include 379 thousand from ischemic heart disease, 165 thousand from lower respiratory tract infections, 36.9 thousand from asthma and 214 thousand from lung cancer (Öberg, Jaakkola, Woodward, Peruga, & Prüss-Ustün, 2011). Of all deaths attributable to SHS, 28% occur in children, and 47% in women. Additionally it amounts to about 10.9 million disability-adjusted life years (DALYs), for the sum of years of potential life lost due to premature mortality, and the years of productive life lost due to disability (WHO, 2011).

In 2012 more than a quarter of the adult Portuguese population smoked (Tables 1 and 2).

Table 1: Portuguese prevalence trend of tobacco smoking among adult (adapted from WHO, 2015b).

		Current use		**Daily use**	
Survey name	Year	**Men**	**Women**	**Men**	**Women**
Inquérito Nacional ao Consumo de Subs. Psicoactivas	**2012**	**35**	**18**		
Inquérito Nacional ao Consumo de Subs. Psicoactivas	2007	40	19		
National Health Survey	2005/06	31	12	**28**	**11**
Inquérito Nacional ao Consumo de Subs. Psicoactivas	2001	40	18		
National Health Survey	1998/99			29	8

The average smoker starts at age 16 and, by the age of 20, becomes a regular smoker. The prevalence is higher in men (35%) than in women (18%). Nonetheless, in the last years the trend is decreasing in men and increasing in adult women. Lisbon and Tagus Valley region have the highest smoking prevalence, 33% (DGS, 2014b).

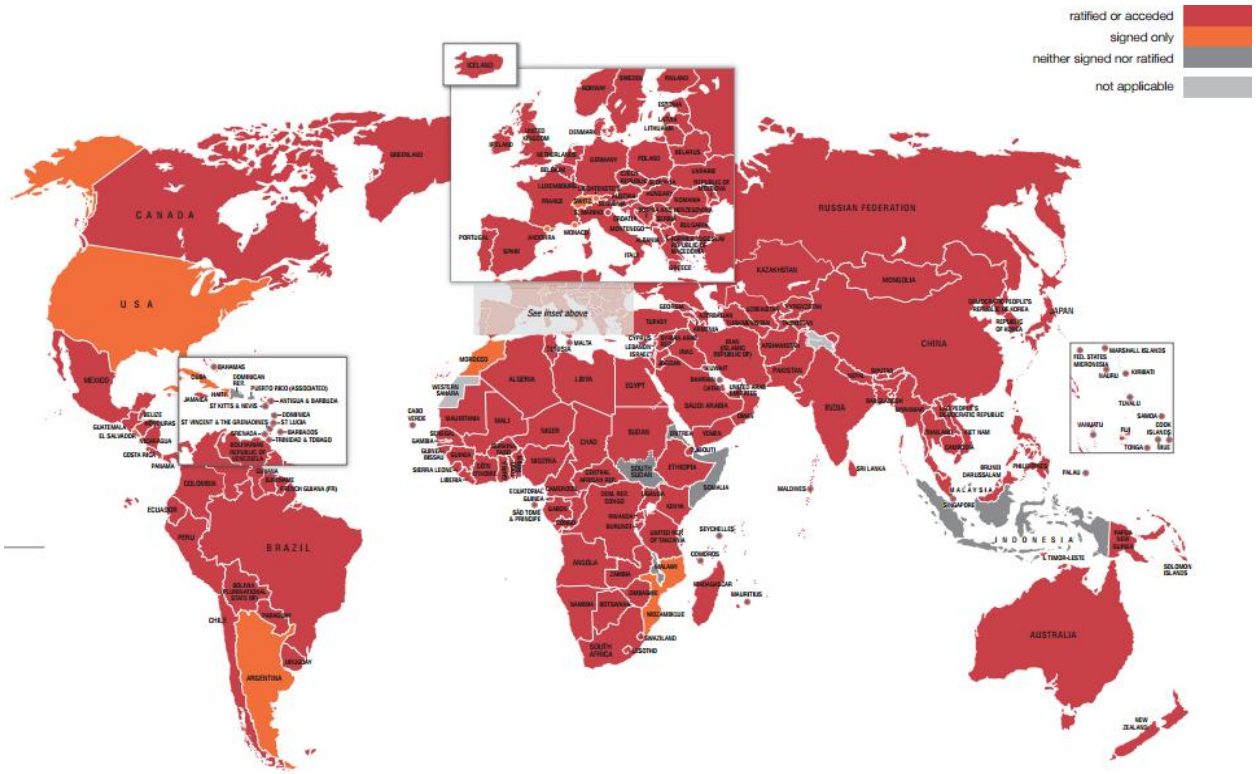
Table 2: Portuguese prevalence trend of tobacco smoking among youth (adapted from WHO, 2015b).

		Current use		**Daily use**	
Survey name	Year	**Boys**	**Girls**	**Boys**	**Girls**
Estudo sobre o Consumo de Álcool, Tabaco e Drogas	**2011**	**17**	**17**		
Health Behaviour in School-aged Children	2010	11	10	**7**	**6**
Health Behaviour in School-aged Children	2002	18	26	13	20

Every year, smoking is responsible for 11.8 thousand deaths, more than 11% of all Portuguese fatalities. It includes the death of 373 men and 472 women non smokers, mostly due to cardio-vascular disease (DGS, 2014b). Impressively, almost a quarter of the population are exposed to SHS at home. Children and young adults are the most affected, accounted by 650 thousand and 939 thousand, respectively. It is noteworthy that upper-income families have almost double the number of self reported passive smokers (29%), compared with lower income families (16%) (Pereira et al., 2013). Additionally, in Portugal, tobacco exposure is the second leading cause of DALY in men and the 7th in women.

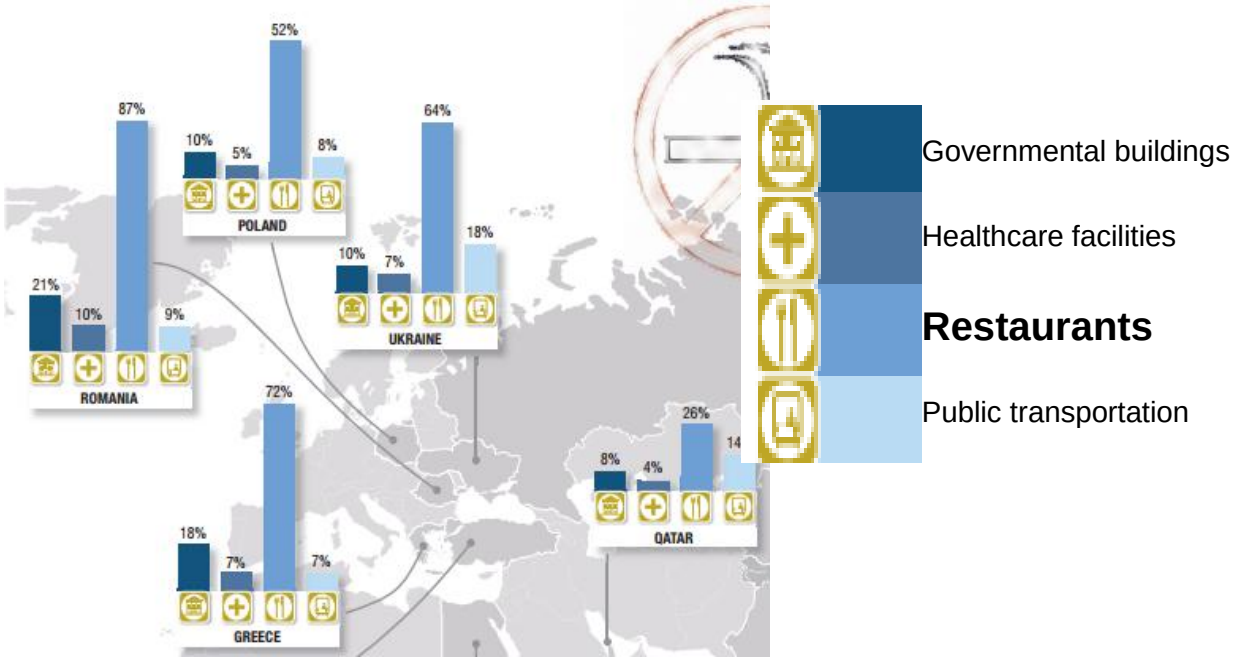
Fortunately, there are comprehensive, evidence-based and cost-effective means to combat this deadly tobacco epidemic (WHO, 2003). Accordingly, in 2003 WHO created a framework with demand reduction provisions. Portugal, together with other countries, joined the initiative (Figure 1). Still, tobacco smoke remains an important hazard for people who work, especially in accommodation and food services (NIOSH, 2015).

Figure 1: WHO Framework convention on tobacco control, as of 4 December 2014 (as is in WHO, 2015).



In fact, restaurants are among the public places with massively higher prevalence of exposure to SHS in the entire world (Figure 2).

Figure 2: Prevalence of exposure to secondhand smoke in public places in some countries, between 2008-2013 (adapted from WHO,2015a).



The creation of smoke-free public places and workplaces, is one of the most commonly established measure at the highest level of achievement (WHO, 2014b). Until 2012, 32 countries passed complete smoking bans covering all work places, public places and public transportation means (WHO, 2014a). Conversely, in Portugal, smoke-free legislations include a number of exceptions and exempt certain venues.

In order to evaluate the effectiveness of the preventative measures undertaken after the 37/2007 tobacco law, the present PhD project was created. *Translating proteomics into environmental health* mostly aims the *biomarkers discovery for tobacco smoke-induced biological damage.*

Proteomics is the large-scale study of proteins, the vital parts of all living organisms. The term "*omics*" was coined after genomics, the study of the genes.

Protein ubiquity, makes proteomic studies a long and successful record in many different fields, sample types and across a diverse range of indications (NCBI, 2015). Nevertheless, these studies are mostly promising and applicable in biomedicine, as they can reflect the biological function, pathways, activities, sub cellular distributions, as well as molecular modifications (Pandey & Mann, 2000). In fact, almost all proteins are modified from their initial translated state. Surprisingly, there are roughly 40 times more proteins than protein-coding genes (Liang, 2013). Hence, posttranslational modifications (PTM) are foremost important to consider, as they vary with time and distinct circumstances, such as exposure to tobacco smoke and stress (Abhilash, M., 2008).

All proteomic technologies rely on the ability to separate proteins from a complex mixture (Abhilash, M., 2008). The chemical properties of a protein, such as their relative mass and isoelectric point (PI), enable their separation in one (1DE) or two electrophoresis dimensions (2DE), respectively. Gel-based electrophoresis, include different staining techniques. Two-dimensional difference gel electrophoresis (2D-DIGE), is an advanced version of classical 2DE, where the gels are stained with a fluorescent dye before the run. Then protein spots are analyzed and compared in a scanned-gel image, aided by specific software tools. Differences in the spot shades translate in a different relative quantification, for example when a protein spot is compared between a specific disease condition and a healthy control (Twyman, 2013). Gel-free methods are usually more expensive and include various tagging or chemical

modification methods. Antibody-based assays can also be used, but are unique to one sequence motif (Abhilash, M., 2008).

Chromatography, is other suitable technique for protein or peptide separation, based in their chemical affinity for two phases, the stationary and the moving phase. Reversed-phase liquid chromatography, is usually performed under high pressures (HPLC) and involves the transient adsorption of elements to the stationary phase matrix. Then multiple fractions are produced by gradient elution, ranging from the more hydrophilic to the higher hydrophobic peptides or proteins (Twyman, 2013).

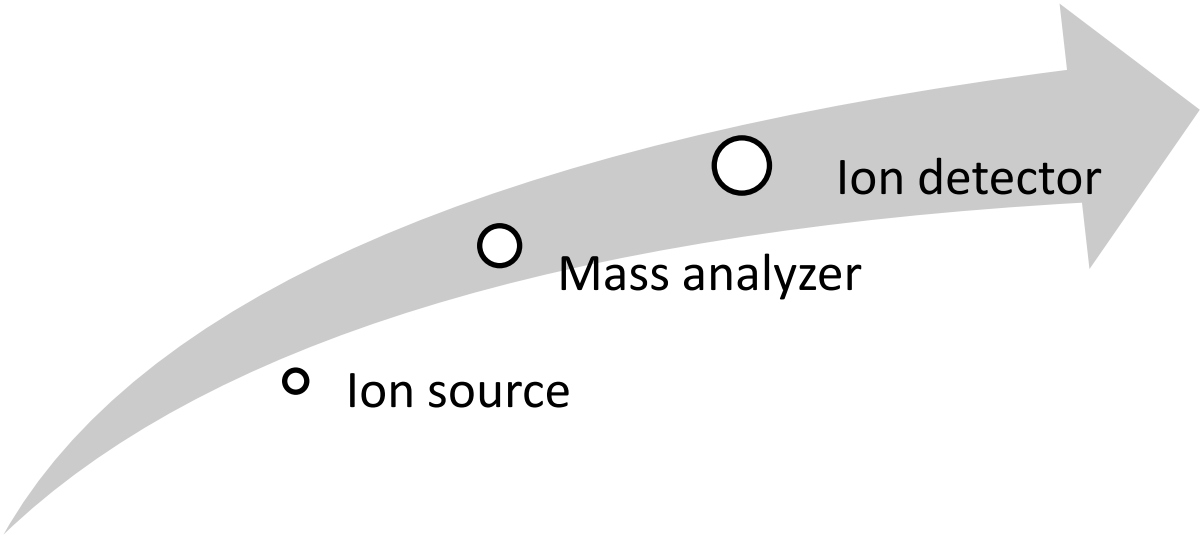
Reverse phase liquid chromatography or 2-DE are frequently combined with Mass spectrometry (MS) technique. MS high sensitivity, specificity and throughput in protein characterization, has become a mainstream and dominant analytic tool (Twyman, 2013). In fact, recent advances in MS based technologies, are coupled with key advances in understanding molecular mechanisms in disease, and novel entry points for therapeutic approaches (Kessler, B, 2013). As a results, more and more investigators develop MS-based proteomics in clinical research to discover, validate and implement biomarkers, as well as to decipher biological processes (Witze, Old, Resing, & Ahn, 2007; C. C. Wu, MacCoss, Howell, & Yates, 2003).

MS separates peptides and proteins according to the mass to charge ratio (m/z) and hence, allow their determination and characterization in multiple sample types.

Low-throughput sequencing methods, through Edman degradation, were replaced by high-throughput MS based proteomic techniques (Edman, 1949). The later, usually includes *de novo* sequencing or peptide mass fingerprinting (PMF), a list of peptide mass values obtained from an *in silico* digestion of a protein by a specific enzyme (Lu & Chen, 2004; Pappin, Hojrup, & Bleasby, 1993).

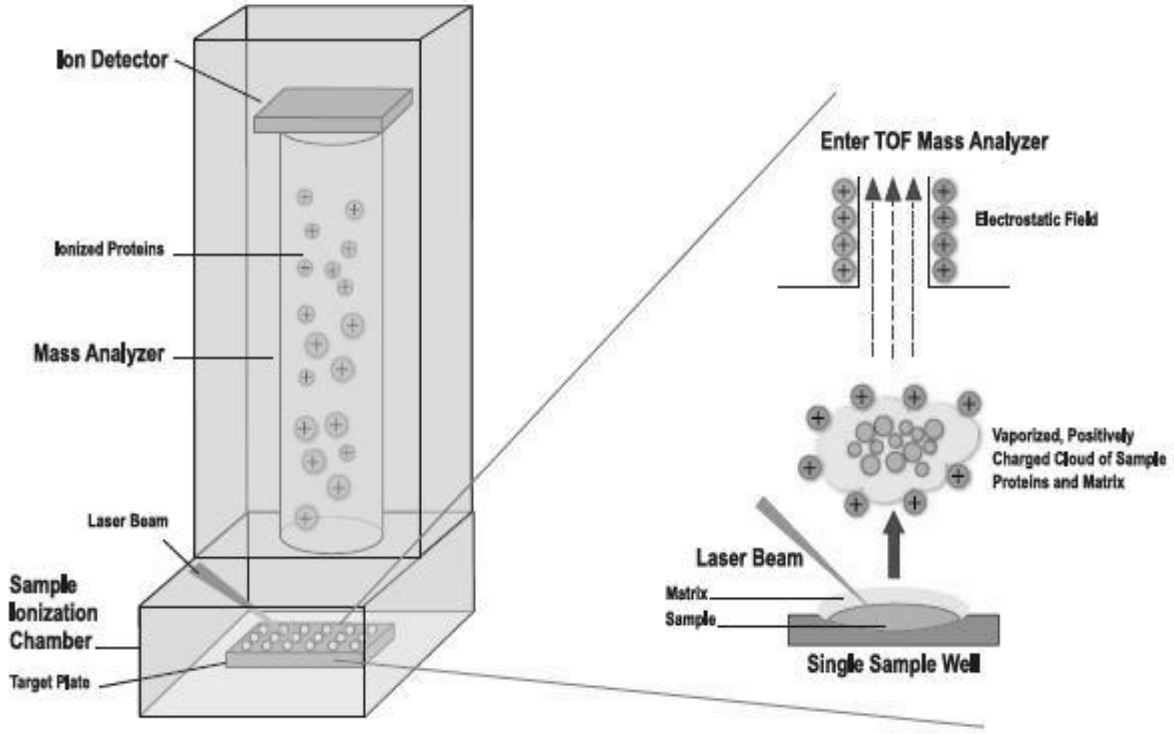
A Mass spectrometer has three main components, namely the ion source, the mass analyzer and the ion detector Figure 3.

Figure 3: Three main components of a mass spectrometer, the ion source, where ions are generated and then separated in the mass analyzer, while they fly towards the ion detector.



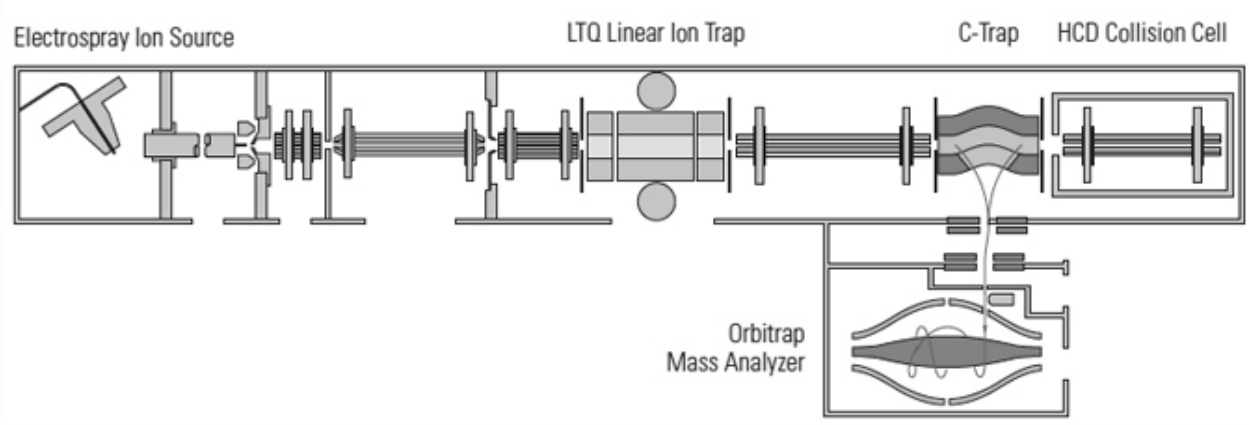
The advent of Nobel awarded soft ionization techniques, in the late 1980s, such as electrospray ionization (ESI) and matrix-assisted laser desorption/ ionization (MALDI), started a technological MS-revolution and became the preferred ionization methods in proteomics (Dole et al., 1968; Gogichaeva, Williams, & Alterman, 2007; Karas, Bachmann, & Hillenkamp, 1985). As for the analyzers, there are five main types, ranging from the more simple quadrupole (Q) and time-of-flight (TOF), to the sophisticated ion trap (IT) and the Fourier-transform ion cyclotron resonance (FT-ICR) and the latest developed Orbitrap (Comisarow & Marshall, 1996; Cotter, Woods, & Cornish, 1994; Hu et al., 2005; Schwartz, Senko, & Syka, 2002). Each analyzer has its own strengths and weakness in terms of accuracy, sensitivity and resolution. Nevertheless, their combination in powerful hybrid instruments, can overcome these limitations. The MALDI -TOF/TOF high-resolution tandem mass spectrometer, combines the advantages of high sensitivity for peptide analysis, with high-resolution and comprehensive fragmentation information (Medzihradszky et al., 2000). The spectrum acquisition, is usually preceded by 2-DE separation technique, spot picking from 2D gels, in-gel digestion and sample preparation on MALDI plates (Suckau et al., 2003).

Figure 4: MALDI-TOF scheme: the sample is ionized by laser irradiation, then the ions are desorbed from the MALDI plate and accelerated into the TOF analyzer. Ions are separated based on their flight time until they finally reach the detector (as is in Theel, E.S., 2013)**.**



The FT hybrid, in Figure 5, combines a linear Ion trap and an Orbitrap mass analyzer (LTQ-Orbitrap), an electrostatic trap where injected ions orbit around an axial electrode (Makarov, 2000). In practice, such analyzers are commonly coupled to liquid chromatography, and measurements are made for transient signals (Makarov, Denisov, Lange, & Horning, 2006).

Figure 5: Scheme of the hybrid linear ion trap -orbitrap mass spectrometer: where both mass analyzers are combined to enhance peptide or protein detection (as is in Thermo F. S., 2015).



Tandem MS analysis (MS/MS), relies on algorithms that match the experimentally obtained MS-sequence, with the peptide and protein sequences stored, in constantly updated databases. These bioinformatic tools, also allow functional assignment of protein domains and prediction of their functions (Peng et al., 2014; Webb-Robertson & Cannon, 2007).

Therefore, mass spectral-based proteomic technologies, are ideally suited for the discovery of protein biomarkers; particularly in this case where there is no prior knowledge of quantitative changes in specific proteins. Aided by proteomic strategies, this project unravels the SHS induced effects in restaurant workers, after the 37/2007 law. I firmly believe that the disclosure of these molecular mechanisms, together with their key intervenient, the protein biomarkers, is crucial to revert decades of millions of priceless losses due to involuntary exposure to tobacco smoke.

1st study

Occupational exposure to environmental tobacco smoke: a study in Lisbon restaurants

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ABSTRACT

Environmental tobacco smoke (ETS), also known as secondhand smoke (SHS), is a major threat to public health and is increasingly recognized as an occupational hazard to workers in the hospitality industry. Therefore several countries have implemented smoke-free regulations in public places, such as restaurants and bars. In Portugal, since 2008, legislation partially banned smoking in those places but air quality data is lacking until now.

The objective of this study was to assess levels of SHS pollution by monitoring indoor fine particles ($PM_{2.5}$) in several restaurants in Lisbon. Occupational exposure to

SHS among these restaurant workers was also monitored through urinary cotinine concentration.

The results showed that the $PM_{2.5}$ median level in smoking designated rooms was 253 µg/m^3, eight times higher than the levels recorded in Canteens or Outdoor. Among locations where smoking is not allowed, the Non-smoking Designated rooms exhibited $PM_{2.5}$ median level higher than smoke-free locations and almost three times above (88µg/m^3) those found in Canteens. Urinary cotinine concentrations were significantly higher in non-smoker employees working in smoking designated rooms, which confirm their exposure to SHS. In addition, the proportion of smokers in the room was found significantly positive correlated with $PM_{2.5}$ concentrations and cotinine. Moreover, even using reinforced ventilation systems, it is still not enough to protect workers from SHS exposure.

These findings demonstrate that the partial smoking ban legislation in Portugal provides no protection to employees working in those venues.

INTRODUCTION

Environmental tobacco smoke (ETS) or secondhand smoke (SHS) is a complex mixture of the gases and particles emanated from the burning end of a cigarette and exhaled mainstream smoke. Particles emitted from burning cigarettes are mainly fine particles with aerodynamic diameter smaller than 2.5 µm ($PM_{2.5}$) and include some of the most toxic compounds in this mixture (Klepeis, Apte, Gundel, Sextro, & Nazaroff, 2003; J. Repace, Hughes, & Benowitz, 2006; Weber & Grandjean, 1987). Given its lowermost diameter, $PM_{2.5}$ can be inhaled deep into the lungs and may cause an array of adverse health effects, including lung cancer and heart disease in adults, exacerbation of asthma and other diseases in children and infants (IARC, 2004; Moritsugu K.P., 2006).

According to the World Health Organization (WHO), it is estimated that about one third of adults worldwide are regularly exposed to SHS (WHO, 2009). In the European Union, 14% of non-smokers are exposed to other people's tobacco smoke at home, and a third of working adults are exposed to SHS at the workplace causing about 7.6 thousand deaths per year. In recognition of the health risks posed by SHS, as is also the case for outdoor air particle pollution, the WHO has encouraged countries to expand

the adoption of smoke-free policies as part of the Framework Convention on Tobacco Control (US-EPA, 2004; WHO, 2009).

Considering that SHS is the major and most disseminated indoor air contaminant, and bearing in mind its adverse health effects, a number of countries have moved to ban smoking in public places, including the hospitality industry (Menzies, 2011). Within such venues, smoke-free laws have contributed to a drastic reduction of $PM_{2.5}$ as shown by several studies (Gorini et al., 2008; Hyland, Travers, Dresler, Higbee, & Cummings, 2008; J. Repace, 2004; J. Repace et al., 2006; Semple, Creely, Naji, Miller, & Ayres, 2007). This indoor air quality (IAQ) parameter, although not tobacco-specific, is a good indicator of indoor air pollution attributed to smoking behaviour (J. Repace et al., 2006; Wallace, 1996).

Although indoor $PM_{2.5}$ monitoring is easy to perform and can provide relatively accurate real-time measurements, the optimal assessment of human exposure to SHS can be performed by monitoring cotinine. This metabolite of nicotine can be measured by analyzing physiological fluids (*e.g.* urine or saliva) of exposed persons correlating with smoke inhalation (Benowitz, 1999; Haufroid & Lison, 1998). In two recent studies, smoking ban led to a significant decrease of urinary cotinine among non-smoking workers contemporaneously with drastic reduction of fine particles (Semple et al. 2007[a]; Valente et al. 2007).

In Portugal, the current legislation (Law 37/2007) states that bars and restaurants smaller than 100 m^2 could be either smoking or smoking-free venues, as long as reinforced ventilation systems are implemented. For establishments bigger than 100m^2, it can be created a smoking section (up to 40% of the total area) physically separated and/or autonomously ventilated. As a result, and given known national smoking prevalence's of 30.6% for men and 11.6% for women over 15 years of age, exposure to SHS can still be an important public health issues affecting hospitality venues' employees (Precioso et al., 2009).

To our knowledge, there are only two studies reporting levels of indoor SHS pollution of public places in Portugal based on both nicotine and $PM_{2.5}$ levels indoors, both performed before implementation of the law 37/2007 (Hyland et al., 2008; Precioso et al., 2007). Therefore, the goals of this study were to assess SHS pollution in Lisbon hospitality venues, mainly through determination of indoor $PM_{2.5}$ levels, as well as

employee's SHS exposure through measurement of urinary cotinine concentrations. In addition, we also explored other aspects that could act as protective factors regarding SHS exposure.

MATERIAL AND METHODS

Restaurants

Among leisure establishments in Lisbon, restaurants and discotheques were pre-selected based on a convenience sample. Accordingly, 58 main venues' owners were invited to participate in the study by letter and personal approach. After detailed information of project goals, 25 agreed to participate, all restaurants. Twelve restaurants were entirely smoke-free, another 12 contain both smoking and non-smoking rooms, and one was entirely designated for smokers. Restaurants were then classified as non-smoke - SFre (n=12), smoke – Sre (n=1) or mixed (n=12) restaurants with both smoking (Sro, n=12) and non-smoking rooms (NSro, n=12).

Given that canteens located in public institutions have been places free of smoke for several years (Law 226, 1983), four canteens (Cant., n=4) were included in the study as $PM_{2,5}$ reference of non-smoking areas.

Considering the fact that $PM_{2.5}$ can penetrate indoors (Wallace, 1996), its levels outside the restaurants were also studied. Due to meteorological and logistical constraints only 16 out of the 25 outdoor sites were monitored (outdoor – O; n=16).

In short, there were 41 indoor areas to monitor (in 29 establishments), grouped into smoking designated areas - SDA (Sro and Sre) and non-smoking designated areas – NSDA (NSro, SFre and Cant) and 16 outdoor.

Data Collection

Sampling events occurred during the period expected to provide the highest patron loads using data logging instruments in different restaurant areas. To avoid any change in restaurant's routine, the instruments were placed in a suitable location assuring the measurements were representative and away from any other potential pollutant source. For establishments with both non-smoking and smoking rooms, measurements were made alternately between the two rooms using the same available

data logging instruments. Monitoring occurred in intervals of around 20 minutes covering a time span of at least one and a half hours.

IAQ parameters

Respirable particle ($PM_{2.5}$) concentrations were measured with Hazdust EPAM5000 particle monitor (SKC Inc.), operating according to ISO7708, within the concentration range of 0.001-20 mg/m^3; precision of ± 0.003 mg/m^3. This equipment measured $PM_{2.5}$ in micrograms per cubic meter ($\mu g/m^3$), every ten seconds. Particle analyzer was zeroed in accordance with manufacturer's instructions before and after each sampling event.

Carbon dioxide (CO_2) and carbon monoxide (CO) concentrations were monitored every ten seconds, using a Photoacoustic Multi-gas Monitor Type 1312, INNOVA, Air Tech Instruments. It measured CO_2 and CO concentration in mg/m^3. The gas analyzer was calibrated in accordance with manufacturer's instructions. The EPAM5000 particle monitor was paired with INNOVA gas analyzer, and the same sampling strategy was used for CO_2, CO and $PM_{2.5}$ concentrations.

Additional information regarding space, ventilation characteristics and the number of clients were also recorded whenever possible. Given the absence of exact area information for most of the establishments, the total guest capacity was considered an indirect indicator of this parameter assuming the same density of furniture. We also recorded the time of the week (work or leisure period), the hour/meal (lunch or dinner) and also the season (cold or warm period), since the study occurred between 2009 and 2011. The number of clients and smokers at each restaurant was registered in the beginning and the end of each monitored period.

Recruited employees

Ethics Committee approval for this study was secured from Nation institute of Health Dr. Ricardo Jorge's Ethics Committee, Lisbon. From the twenty-nine locations, a total of 97 employees were recruited for SHS exposure evaluation. Selection took no specific account of gender or ethnic background and included subjects with more than 18 years old, who work more than nine hours per week and for at least one month at the current workplace. After informed consent, an interview was completed for each subject to access demographic and personal lifestyle data, mainly to evaluate smoking habits or

subjective exposure to passive smoke. Eighty-seven, out of all employees recruited, consented to provide urine sample contemporaneous with the $PM_{2.5}$ measurements. Each urine sample was labeled and stored at -20°C until further analysis.

Urinary cotinine analysis

Urinary cotinine concentrations were measured using Gas Chromatography Mass Spectrometry (GC-MS) as described before (Hemmersbach & de la Torre, 1996). Cotinine concentrations were expressed as the average of two replicates. The quantification limit (QL) for cotinine was estimated to be five ng/mL.

Statistical analysis

For each site, the maximum levels of measured $PM_{2.5}$, CO_2 and CO parameters were considered assuming the worst scenario of exposure. Cotinine average concentrations below the detection limit were replaced by 0.1 ng/mL for analysis purpose. Quantitative data are described by Mean, Standard Deviation (SD), Median, Minimum (Min) and Maximum (Max) while qualitative data are described in absolute values (and the respective percentage).

Statistical analysis was performed in SPSS v.17.0, Excel and Access from Microsoft® Office 2007 and based on two-sided tests with a 0.05 significance level (α).

Differences between the sampling sites in what concerns particulate and gas phase were assessed based on Mann Whitney paired test for each pair of sites (with Bonferroni adjustment of the significance level) and Kruskal-Wallis tests, respectively.

Two independent analyses were performed to explore differences on urinary cotinine levels collected from employees working on SDA or NSDA: one for Smokers (t-test) and another one for Non-Smokers (Mann Whitney U).

To explore possible influential factors on measured $PM_{2.5}$ levels and Cotinine concentration making use of information regarding establishment features and workers' personal data, Spearman's correlation and the odds ratios (OR) were performed for quantitative and qualitative data, respectively. The OR was calculated mainly for all the significant variables after the Fisher's Exact Test or Pearson Chi-Square test. To convert Cotinine and $PM_{2.5}$ levels into dichotomous variable, the following thresholds were considered, respectively: five ng of cotinine per mL of urine and 73 µg of $PM_{2.5}$ per m^3 (the average of $PM_{2.5}$ levels in both canteens and outdoor).

Results

Indoor air quality analysis

The summary of measured IAQ parameters is presented in Table 3.

Table 3: Indoor air quality parameters measured by sampling site category.

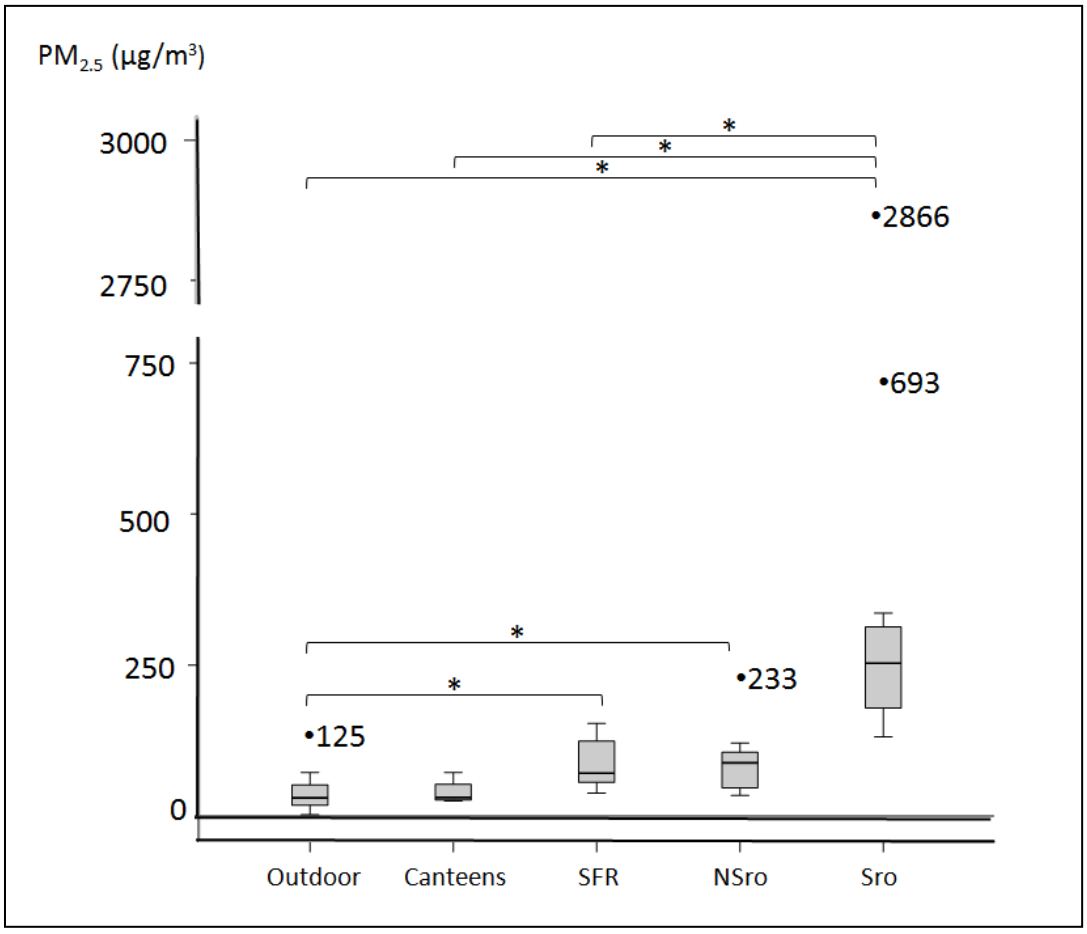
		Sro (N=13)	NSro (N=12)	SFre (N=12)	Canteens (N=4)	Outdoor (N=16)
Fine Particulate Matter $PM_{2.5}$ ($\mu g/m^3$)	Median	253	88	66	30	30
	Min	164	34	38	25	2
	Max	2866	131	153	72	125
	Mean	476	81	84	40	37
	SD	714	34	40	22	31
Carbon monoxide (mg/m^3)	Median	1.6	1.1	0.9	0.9	
	Min	0.8	0.7	0.5	0.5	
	Max	9.9	3	4	11	
	Mean	2.4	1.4	1.3	3.3	
	SD	2.4	0.8	1.0	5.1	
Carbon dioxide (mg/m^3)	Median	1620	1725	1580	1780	
	Min	1310	1330	1020	1630	
	Max	4130	2640	2690	3870	
	Mean	1925	1860	1766	2265	
	SD	769	362	575	1073	

Sro, smoking rooms; Nsro, non-smoking rooms; Sfre, smoke-free restaurants.

Among all the locations studied, $PM_{2.5}$ median levels were significantly higher in smoking rooms (Sro), ranging from concentrations of 164 to 2,866 $\mu g/m^3$ (Figure 6). Moreover, the median levels of fine particles were higher in non-smoking rooms (Nsro, 88 $\mu g/m^3$), above those found in smoke-free restaurants (Sfre 66 $\mu g/m^3$) and both canteens (Cant) and the exterior (E) (approximately 30 $\mu g/m^3$). $PM_{2.5}$ levels were also found significantly ($p<0.005$) higher in NSro and SFre when compared with the E.

As shown in Table 3, CO_2 and CO levels were also monitored and no significant differences were observed between the locations studied ($p<0.05$). Nevertheless, the highest median level of CO was observed in Sro (1.6 mg/m^3) while in Sfre and Cant were the lowest (0.9 mg/m^3). Still, Cant recorded the highest value and the higher range of CO concentrations (from 0.5–11 mg/m^3) followed by Sro (from 0.8 to 9.9 mg/m^3). The highest median level of CO_2 was obtained in Cant (1,780 mg/m^3), followed by NSro (1,725 mg/m^3), while SFre recorded the lowest one (1,580 mg/m^3). In Cant, as in Sro, we registered the most scattered and higher maximum levels of CO_2.

Figure 6: Fine particulate levels ($PM_{2.5}$) by site category.



Sro, smoking rooms; Nsro, non-smoking rooms; Sfre, smoke-free restaurants; *p-value<0.005

An exploratory analysis of IAQ parameters measured in the 29 locations was performed based on some of their characteristics, as depicted in Table 4.

Table 4: Characteristics of the sampling locations, according to area category.

Qualitative data						
		N	**SDA (%)**		**NSDA (%)**	
Ventilation	**Hybrid or mechanical**	32	13 (32)		19 (46)	
	Natural	9	0 (0)		9 (22)	
Physical separation and/or autonomous ventilation between Sro and NSro	**Yes**	40	13 (32)		27 (66)	
	No	1	0		1 (2)	
Communication between the room and exterior	**Yes**	10	4 (10)		6 (15)	
	No	31	9 (22)		22 (53)	
Total Rooms		13	13		28	
Quantitative data						
		Median	**Min**	**Max**	**Mean**	**SD**
Room capacity	**SDA**	32	20	80	37	16
	NSDA	51	8	156	56	28
Proportion of smokers in the room	**SDA**	40	19	100	45	25
Proportion of persons in the room	**NSDA**	63	7	100	62	26
	SDA	59	20	100	66	27

SDA, smoking designated area; NSDA, non-smoking designated areas.

Regarding quantitative data, $PM_{2.5}$ levels were significantly correlated with the proportion of smokers in the room (Spearman's correlation coefficient - CC=0.77, p=0.00), room capacity (as an indirect measure of room area; Spearman's CC=-0.60, p=0.00) and CO levels (Spearman's CC=0.35, p=0.03) (Table 5). In what concerns qualitative data, a significantly higher proportion of restaurants presents higher $PM_{2.5}$ levels when a ventilation system is installed (either mechanical or hybrid) compared to

unventilated places (OR=5.11; p=0.05). Since this might be explained by the fact all places where smoking is allowed are ventilated, in contrast with smoke-free restaurants, we performed an additional analysis only for mixed restaurants. Although not statistically significant, the results showed that a higher proportion of those restaurants recorded higher $PM_{2.5}$ levels when procedures to restrict SHS pollution are taken. These procedures include autonomous ventilation and/or physical separation, although not tight, between Sro and NSro, and any sort of room communication with the outdoor.

Besides fine particles, CO levels were also significantly correlated with the proportion of smokers in the room (Spearman's r=0.37; p=0.02) (Table 5). In contrast, none of the referred factors were significantly correlated with levels of CO_2 measured in the 29 places.

Table 5: Factors that were found to influence indoor air quality parameters and urinary cotinine levels, in studied locations. Statistical analysis was based on Spearman's correlation.

		$PM_{2.5}$ ($\mu g/m^3$)		**Room Capacity**		**Proportion of Smokers in the room**	
		CC	p	CC	p	CC	p
Indoor Air Quality Parameter	**$PM_{2.5}$** ($\mu g/m^3$)			-0.6	0	0.77	0
	CO (mg/m^3)	0.35	0.03			0.37	0.02
Cotinine		0.45	0			0.43	0

Spearman's correlation coefficient (CC) and p-value (p).

Cotinine analysis

SHS exposure was also evaluated based on cotinine levels in workers' urine from those 29 establishments under study. In total, 96 workers were recruited, the majority being males (75%) with an age close to 40 (average value). Most reported working at least 40 hours per week (Supplementary information: Table 16). From this population, 87 workers agreed to provide a urine sample for cotinine analysis, which corresponded to one sample to five in maximum from each establishment. Given that

one worker, self-reported as non-smoker, exhibited urinary cotinine levels (1,806 ng/mL) in the range of concentrations known for smokers (Jarvis, Tunstall-Pedoe, Feyerabend, Vesey, & Saloojee, 1987), this data was excluded from our analysis. Concerning smoking status, 62 of these workers were non-smokers: 27 were working on smoking-designated areas (SDA) and thus exposed to SHS while the remaining were working on non-smoking areas (NSDA). Ten of the smoker workers were also working on NSDA (Table 6).

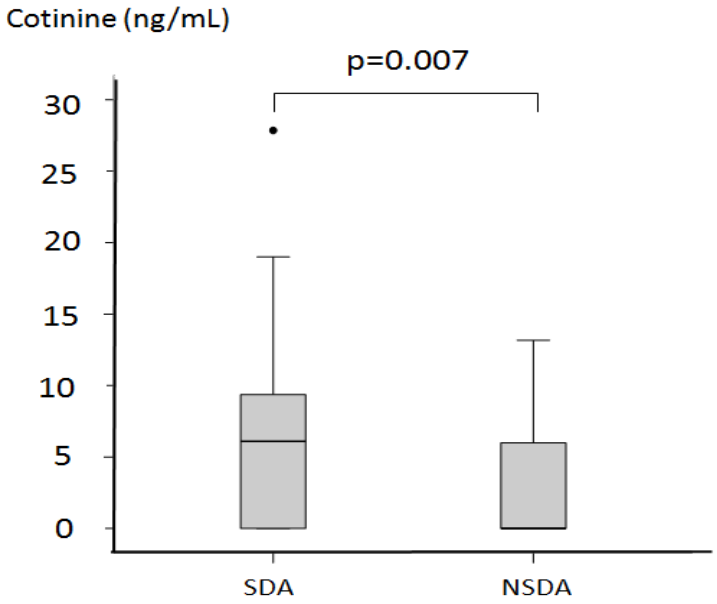
Table 6: Cotinine levels in workers' urine by sampling area category

Urinary cotinine in workers (ng/mL):		**SDA**	**NSDA**
Non smokers * N=62		N=27	N=35
	Median	7	<QL
	Min	<QL	<QL
	Max	27	19
	Mean (SD)	7.9 (6)	<QL (3.6)
Current smokers N=24		N=14	N=10
	Median	1820	1580
	Min	265	236
	Max	3124	2500
	Mean (SD)	1820 (838)	1417 (792)

* Subjects who never smoked or did not smoke for at least 6 months prior to sample collection.

As expected, the urinary cotinine levels were found much higher in smoker workers than in non-smoker ones. Moreover, among smokers, median levels of urinary cotinine in exposed workers were higher (1,820 ng/mL) than in those not exposed (1,580 ng/mL), although not statistically significant. Importantly, among non-smokers, the median cotinine levels measured in exposed workers (7 ng/mL) were significantly higher than those found in unexposed ones (below QL) (Figure 7).

Figure 7: Urinary cotinine levels in non-smoker workers by area category. Statistical analysis was based on Mann-Whitney significance test.



SDA, smoking designated area; NSDA, non-smoking designated areas.

A exploratory analysis on the cotinine levels found in workers' urine was also performed based on the corresponding establishment's characteristics, described in Table 4. In accordance with above results, urinary cotinine concentrations were significantly correlated with $PM_{2.5}$ (Spearman's CC=0.45; p=0.00) and also with the proportion of smokers in the room (Spearman's CC=0.43; p=0.00) (Table 5). Furthermore, we found that there is a higher probability (more than five times) of detecting high cotinine concentrations in non-smoker workers' urine when they were working in SDA compared to those working in NSDA (OR=5.40; p=0.00).

Discussion

The present work demonstrated high indoor contamination of fine respirable particulate $PM_{2.5}$ in all studied Lisbon restaurants where smoking is allowed and/or have dedicated rooms to smoking, in comparison with smoking-free restaurants or canteens.

The $PM_{2.5}$ levels found in Sro ranged between 164 to 2,866 $\mu g/m^3$. To our knowledge there are no studies assessing this parameter in Portuguese restaurants besides the one from (Hyland et al., 2008). This study presents data collected in

restaurants located in 32 countries, including Portugal. Comparing to their results, we found $PM_{2.5}$ mean levels around 3 times higher. This result is not surprising given that SHS is the main contributor to indoor $PM_{2.5}$ levels (James L. Repace, Hyde, & Brugge, 2006; Wallace, 1996). In fact we found a significantly positive correlation (Spearman's CC 0.77, $p<0.05$), between higher $PM_{2.5}$ concentrations and the proportion of smokers in the room. Additionally, a negative correlation was found with room capacity, the indirect measure of space area ($p<0.05$). This seems to corroborate results obtained by Hyland and others (Hyland et al. 2008, Karabela et al. 2011), positively correlating higher $PM_{2.5}$ concentrations with smoker density (average number of cigarettes per 100 m^3 of venue air volume) in monitored spaces. The outlier value of 2,866 $\mu g/m^3$ (Figure 1) might be reflecting this association given the fact in this particular restaurant the volume area was extremely small. The other outlier value of 693 might be additionally explained by a factor not explored in this work; the use of furniture and textiles that are difficult to clean, favouring particle accumulation, namely $PM_{2.5}$ (Matt et al., 2011).

Moreover, we observed a significantly positive correlation between higher $PM_{2.5}$ levels and CO concentrations. Despite no significant differences were found across all places studied, the median level of CO was higher in Sro, which suggests a common origin with fine particles: tobacco combustion. Importantly, urinary cotinine levels of non-smoker employees working in smoking-designated rooms were significantly higher (5.6 times) than those measured in urine samples collected from non-smoker employees working in non-smoking designated areas.

Furthermore, employees working in smoking rooms were significantly more likely to experience 5 times greater increase in urinary cotinine levels compared to employees working in similar venues that did not allow smoking. These results demonstrate that workers are effectively exposed to SHS in SDA, inhaling tobacco combustion sub-products.

Since 2006, the Environmental Protection Agency – US EPA – has set that outdoor $PM_{2.5}$ levels of 35 $\mu g/m^3$ should not be exceeded more than once in a year. According to this guideline, which is a measure for reducing the health impacts of air pollution, the referred threshold is associated with 15% higher long-term mortality risk (WHO 2009). In this study, all places where smoking is allowed had $PM_{2.5}$ levels that probably would result in a average exposure over 35 $\mu g/m^3$. Median $PM_{2.5}$ concentration

found in canteens (where smoking is forbidden for a longer time), was similar to outdoor background levels of fine particulate matter (near 30 μg/m^3), which seem to make these places good references for SHS pollution comparisons.

Since most of the recruited restaurants where smoking is allowed also possessed areas reserved for non-smokers, we also monitored SHS pollution in these places (NSro). According to the current Portuguese tobacco legislation, at least 60% of the interior space of the leisure place must be reserved for-non-smokers, separated physically if possible or autonomously ventilated, to restrict SHS pollution. The present study shows a median $PM_{2.5}$ concentration of 88 in these rooms. Analyzing only mixed restaurants making use of data regarding space characteristics and other procedures in use to restrict SHS pollution we observed a positive correlation between higher $PM_{2.5}$ levels and the existence of autonomous ventilation or some sort of physical separation, between Sro and NSro.

Analyzing all establishments, there was a significant higher risk of exhibiting increased $PM_{2.5}$ levels (five times) in restaurants with ventilation systems (either mechanical or hybrid) compared to unventilated.This last observation might result from the fact all mixed restaurants, in contrast with entirely smoke-free places, had ventilation systems, regardless of being independent or shared to both rooms. In a previous study, Repace and co-workers (James L. Repace et al., 2006) while monitoring $PM_{2.5}$ levels in smoking Boston bars provided evidence that ventilation failed to control respirable particulates, although designed to control CO_2 levels for the number of occupants present. Accordingly, regardless of the type of space, the CO_2 median level was above the Portuguese legal limit of 1,800 mg/m^3 (Law 79, 2006).

The fact SHS is not the only source of fine particulate matter indoors should be considered when interpreting this IAQ parameter. Ambient particle concentrations, cooking and candle burning are examples of additional sources of indoor $PM_{2.5}$ (Wallace, 1996). In our establishment population, SFre exhibited median concentration of fine particles close, despite lower, to the median concentration found in NSro. Moreover, this level was found relatively high compared to the public canteens or other smoke-free venues in other countries, as shown by Hyland et al. (2008). This observation can be explained by several factors such as the outdoor communication,

the usage of open fires for cooking, the deposit of particles in the furniture and textiles and the frequency of cleaning procedures.

Despite the fact that fine particles could easily diffuse through windows and doors, our results does not point to a reduction of indoor $PM_{2.5}$ in SHS polluted restaurants depending on opening to outdoor, as observed by Vardavas and co-workers in Greek hospitality venues (Karabela et al., 2011). Accordingly, there was no significant difference in the proportion of sites with higher $PM_{2.5}$ levels between the warm and the cold season, which is when the doors and windows are opened or closed respectively. Therefore smoking with open doors and windows still leads to higher SHS exposure.

Conclusion

This work provides evidence that partial smoking restriction in Portuguese hospitality venues such as restaurants do not sufficiently protect hospitality workers against SHS. Whatever protective measures are used, namely reinforced ventilation systems the exposure to hazard SHS levels remains high in restaurants where smoking is allowed This follows WHO's statements assuming that ventilation systems are incapable of removing tobacco smoke derived particulate matter and abolishing smoke from these establishments is the most effective strategy to reduce SHS exposure (WHO, 2009).

Based on these results, new comprehensive smoke-free legislation is urgently needed in Portugal to fully protect workers from exposure to SHS in restaurants or, broadly, public health in indoor environment.

2nd study

Effects of occupational exposure to tobacco smoke: seeking for a link between environmental exposure and disease

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Abstract

In a previous study, we provided evidences that indoor secondhand-tobacco smoke (SHS) air pollution remains high in Lisbon restaurants where smoking is allowed, regardless of the protective measures used. Here, we are estimating the levels of polyaromatic hydrocarbons associated with the particulate phase of SHS (PPAHs), a fraction that contains recognized carcinogens, such as Benzo[a]pyrene. According to a previous model and our measurements of fine particles ($PM_{2.5}$), we estimated that restaurants' smoking areas could present PPAH levels as high as 110 ng/m^3, a value

significantly higher than that estimated for non-smoking areas (30 ng/m^3) or smoke-free restaurants (22 ng/m^3). The effective exposure/ inhalation of the components of SHS at restaurant' smoking rooms was proved by monitoring the cotinine marker in workers' urine. Considering that all workers exhibited a normal lung function, we aimed to study biochemical and, mostly, molecular changes that could be induced by SHS in those occupationally exposed compared to those unexposed. No significant differences were observed on the levels of two oxidative markers - total antioxidant status and 8-OHdG - measured on plasma and serum collected from workers. However, SHS-exposed employees exhibited higher mean levels of 8-OHdG than those not exposed, regardless of their smoking status. Remarkably, a proteomics approach based on 2D-DIGE-MS was able to identify 9 differentially expressed proteins in depleted plasma collected from SHS exposed workers compared to unexposed ones. Out of these, Ceruloplasmin and Inter-α-trypsin inhibitor heavy chain H4 (ITIH4), two acute-phase inflammation proteins, became prominent. These two proteins presented a high number of isoforms, exhibiting either an increase or a decrease in their abundance associated with the exposure to SHS. A detailed observation of the CP and ITIH4 isoforms, highlighted the fact all high-molecular-weight isoforms suffered a decrease in their abundance and, in parallel, low-molecular-weight isoforms suffered an increase in their abundance. Whether this expression likely SHS-induced profile is due to a specific proteolytic cleavage or an increased instability possibly due to oxidative modifications remains to investigate. Considering that these events precede the onset of the first symptoms of tobacco-related diseases, these findings might contribute for disclosing early SHS-induced pathogenic mechanisms and might constitute a useful tool for monitoring the effects on occupational exposed individuals such as those working on hospitality industry.

Introduction

Tobacco smoke kills nearly a million people and causes hundreds of billions of dollars of economic damage worldwide each year (WHO, 2011). It is associated with several diseases, most notably respiratory-related diseases such as Chronic Obstructive Pulmonary Disease (COPD) and lung cancer, but is also associated with heart and cerebrovascular diseases (CDC, 2010); affecting not just sSmokers (S) but also non-smokers (NS) who breathe in secondhand-tobacco smoke (SHS). SHS has

been recognized as a health hazard by environmental, occupational and public health authorities worldwide and owe its hazard to the emission of toxins into the air from burning cigarettes, pipes and cigars as well as exhaled tobacco smoke from smokers (WHO, 2011). Thousand chemical compounds have been identified in SHS, including known carcinogens and hundreds of toxic or irritating substances such as carbon monoxide, benzene, formaldehyde, hydrogen cyanide, ammonia, formic acid, nicotine, nitrogen oxides, acrolein and fine particulate matter (Hoffmann & Hoffmann, 1987). Among the referred carcinogens are Polycyclic Aromatic Hydrocarbons (PAHs), a group of environmental pollutants produced during incomplete combustion of organic materials, including tobacco. At least 539 PAHs and their alkyl derivatives, are known from tobacco combustion and the most dangerous seems to be partitioned into the particulate phase (James L. Repace et al., 2006; St Helen et al., 2012).

Despite all these knowledge, SHS remains a common indoor air pollutant in many countries, especially in public places such as restaurants, bars, nightclubs and casinos. In these places, tobacco-derived Particulate Matter, mostly fine particles ($PM_{2.5}$), and Particulate bounded-PAH fraction (PPAH), among other, can reach very high levels (Bolte et al., 2008; Pacheco et al., 2012; Precioso et al., 2007; James L. Repace et al., 2011). Consistently, we found that SHS pollution in Lisbon hospitality venues was higher in restaurant' smoker rooms compared to any other non-smoking rooms and that their non-smoker employees were occupationally exposed to SHS. This observation was based on the measurement of indoor $PM_{2.5}$ and a metabolite of nicotine, cotinine, in worker's urine. Both markers were positively correlated with the proportion of smoker occupants in those rooms (Pacheco et al., 2012). Thus, the absence of smoke-free policies for the hospitality industry keep these indoor environments as places of likelihood health risk to millions of individuals, particularly their workers.

How chronic exposure to SHS exactly translates into an increased risk of developing chronic diseases remains to be answered. Nevertheless, several evidences suggest that SHS exposure increases oxidative stress, which in turn induces biological damage (CDC, 2010). Consistently, it was proved the presence of large amounts of reactive oxygen species (ROS), namely in the tobacco particulate-phase, which can interact with molecules such as DNA, lipids and proteins (CDC, 2010; Hoffmann &

Wynder, 1986). Ultimately, the accumulation of biological damage in tissues and organs might alter normal physiological activities leading to the onset of chronic diseases.

In this study, we aimed at identifying biochemical and molecular changes induced by occupational SHS exposure, by investigating exposed workers from those previously studied Lisbon restaurants (Pacheco et al. 2012). Here, the workers of these restaurants are detailed demographic and clinically characterized and their plasma and serum measured for total antioxidant status (TAS) and 8-Hydroxydeoxyguanosine (8-OHdG), a marker of oxidative DNA damage, respectively (Howard, Ota, Briggs, Hampton, & Pritsos, 1998; Nakajima et al., 2012). The effect of SHS exposure on plasma proteome was investigated by two-Dimensional Difference In Gel Electrophoresis (2D-DIGE) proteomics approach. Using this proteomic approach we expect to contribute for further discussion about the early molecular responses to SHS exposure that might lead to chronic diseases.

Material and methods

Restaurants

As previously described (Pacheco et al. 2012), monitored restaurants were classified as non-smoke (SFre), mixed restaurants with both smoking (Sro) and non-smoking rooms (NSro). Additionally, we monitored outdoor air (n=16) and three canteens (Cant) located in public institutions. From a total of 29 establishments, 41 indoor areas were monitored and grouped as smoking designated areas (SDA) which included Sro (n=13) and non-smoking designated areas (NSDA) which included NSro (n=12), SFre (n=13) and Cant (n=4).

Monitored indoor parameters

Measurement of indoor $PM_{2.5}$, CO and CO_2 parameters occurred in intervals of around 20 min covering a time span of at least 90 min using data logging instruments. A full description on methods and data collection can be found in Pacheco et al 2012.

PPAH level estimation

Based on previous collected data (James L. Repace et al., 2006), PPAH levels were estimated assuming that there is a negligible difference between $PM_{2.5}$ and $PM_{3.5}$, especially for the submicron SHS aerosol (Klepeis et al., 2003; James L. Repace et al., 2006; J. L. Repace & Lowrey, 1980). According to Repace and co-workers, regression

analysis yields a good linear fit (R>0.85) between hospitality venues PPAH and fine particles measured indoors, subtracted from outdoor levels. Moreover, each parameter strongly correlates with smoker density (J. Repace, 2004; James L. Repace et al., 2011, 2006). Therefore we employed the following equation (Repace 2004):

SHS_{PPAH} (ng/m^3) = 2.054 SHS_{RSP} (µg/m3) – 1.741 (r=0.860),

where SHS_{PPAH} stands for the total indoor PPAH subtracted from the average PPAH background (estimated SHS specific-PPAH) and SHS_{RSP} stands for total indoor respirable/fine particles (RSP) minus the average RSP background (estimated SHS specific-RSP).

Recruited employees

From the 29 locations, a total of 96 employees were recruited. After informed consent, biological samples were collected and an interview was completed to evaluate demographic and lifestyle factors (Supplementary information: Tables 18 and 19). Workers were separated in four study groups according with smoking status and SHS occupational exposure as follows: non-smokers not exposed (NSNE), non-smokers exposed (NSE), smoker not exposed (SNE) and smoker exposed (SE).

Sample collection

Eighty-seven workers consented to provide a urine sample contemporaneous with the $PM_{2.5}$ measurements. Each urine sample was labeled and stored at -20°C until urinary cotinine was further analyzed (Pacheco et al 2012). Eighty-one individuals provided blood samples that were processed in plasma and serum as described elsewhere before storage at -80ºC (Charro et al., 2011).

Respiratory function analysis

Seventy-eight spirometry tests were performed to assess worker's respiratory function. Any pre-existent respiratory disease was excluded through clinical evaluation. A portable spirometer (Vitalograph COMPACT model 6600) was used together with disposable bacterial filters, to avoid cross infection with microorganisms between subjects. Spirometry tests were performed by cardio pulmonology technicians according to the guidelines of the American Thoracic Society (ATS) and the European Respiratory Society (ERS), using the reference values established by the Global Initiative for Chronic Obstructive Lung Disease (GOLD, 2010; M. R. Miller et al., 2005). Spirometric indices studied included i) the Forced Vital Capacity (FVC), which is the total volume of

air that the examined individual can forcibly exhale in one breath, ii) the forced expiratory volume in one second (FEV1), which is the volume of air that the examined individual is able to exhale in the first second of forced expiration and iii) the ratio of FEV1 to FVC (FEV1 /FVC), expressed as a fraction. Values of FEV1, FVC and the ratio are measured in litters and are presented as a percentage of the predicted values for that individual, according to parameters such as race, gender, age and body mass index (BMI).

Analysis of possible SHS-induced biochemical and molecular changes

Total antioxidant status (TAS)

The TAS of worker's plasma was determined by Miller & Rice-Evans method (1997), with an aid of commercially available reagent kit (Randox Laboratories Ltd, Ardmore, UK). In this method, incubation of ABTS [2,2'-azino-bis-(3-ethylbenzthiazoline sulphonate)] with peroxidase and hydrogen superoxide leads to the formation of blue-green-colored cationic radical ABTS 0. A decrease in the color intensity of this radical can be observed when antioxidants are present within analyzed sample. This decline in color intensity was measured spectrophotometrically (UV-Vis Unicam, UV-4) at 600 nm wavelength. The TAS is expressed in mmol/l being the average of two independent experiments.

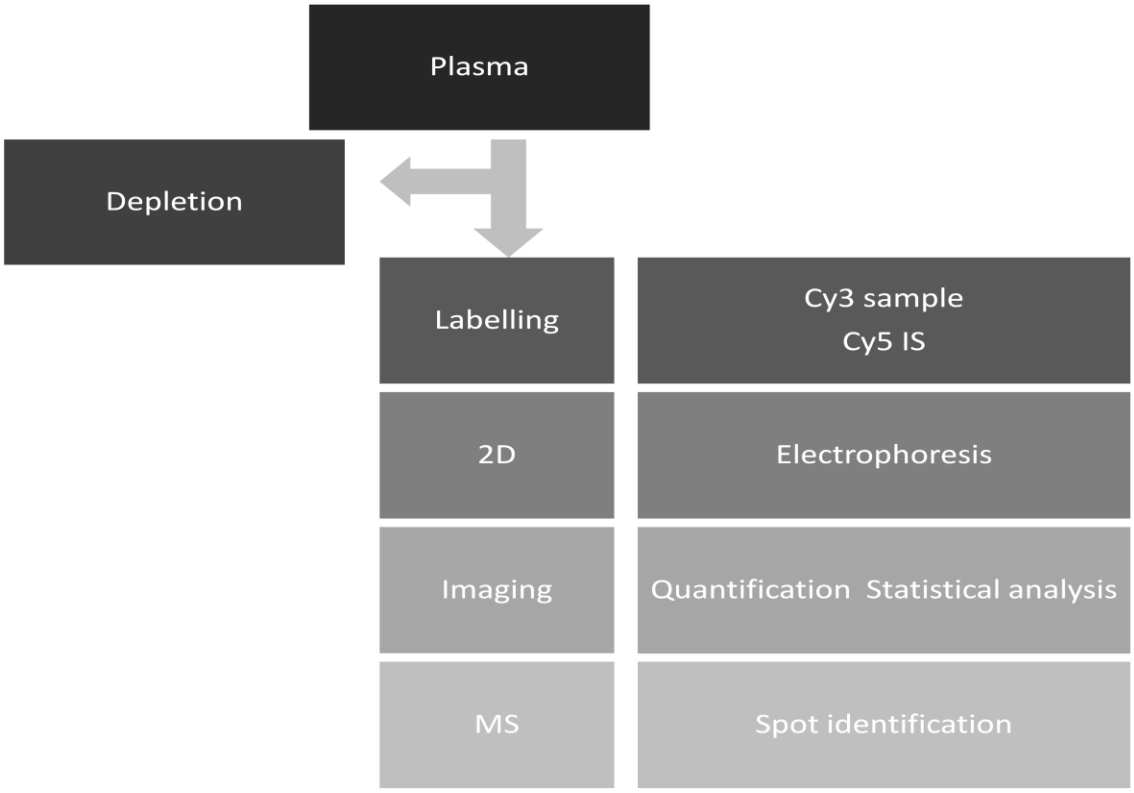
8-OHdG

DNA adduct 8-OHdG was measured in blood serum using an Enzyme-Linked Immunosorbent Assay (ELISA) commercially available (8-Hydroxydeoxyguanosine Check; Japan Institute for the Control of Aging, Shizuoka, Japan) (Saito et al., 2000). The concentration of 8-OHdG is expressed in ng/ml, being the average of two independent experiments.

Proteome analysis

Figure 8 is depicting a simplified workflow of the steps followed for proteome analysis, which are described in detail bellow.

Figure 8: Flow chart of the proteomic workflow: from plasma depletion to DIGE analysis (labeling, 2D electrophoresis, gel images analysis) and finally MS identification of the differentially expressed protein spots.



Sample preparation

Sample preparation for plasma protein analysis was based on a pooling strategy to emphasize proteomic differences among the groups studied, while eliminating potential individual contributions (Charro et al., 2011; Huang, Hinds, Qi, & Prentice, 2010).

Three pools were constructed for each study group (NSNE, NSE; SNE and SE. Each pool was constructed with a similar number of individual samples (the number of smokers limited the dimension of the pools) and its selection was based on several criteria to eliminate potential confounding factors. Differences observed among the pools in terms of DNA repair capacity (unpublished results) were also taken in consideration for possible correlation of DNA damage and protein patterns/damage. DNA repair capacity was measured by the comet assay in leukocytes following a genotoxic challenge (Louro et al. 2011).

Pooled plasma samples were processed using Multiple Affinity Removal System (MARS) spin cartridges (P/N 5188-6560, 0.45 mL resin bed, Agilent Technologies, Palo

Alto, CA) to remove the 14 most abundant proteins (albumin, IgA, IgG, α-1-antitrypsin, transferring, haptoglobin, fibrinogen, α-2-macroglobulin, α-1-acid glycoprotein, IgM, apolipoprotein AI, apolipoprotein AII, complement C3 and transthyretin), according to manufacturer's instructions. Removal of these high abundant species was confirmed by one- dimension sodium dodecyl sulfate-poly acrylamide gel electrophoresis (1D SDS-PAGE) of both bound and un-bound fractions. Un-bound fractions were concentrated and buffer-exchanged to 25 mM of ammonium bicarbonate (AmBic)NH_4HCO_3 by centrifugal filtration using 5 kDa molecular weight cut-offs (MWCO) (Amicon Ultra 4, Millipore) spin concentrators. Protein concentrations were established using a BCA protein assay kit (Pierce). Samples were stored at −80°C until further analysis.

Plasma protein analysis

Analysis of depleted plasma proteins from the four study groups was based on a 2D-DIGE approach, using the CyDye DIGE fluor minimal dyes - Cy3 and Cy5 - from GE Healthcare. Briefly, each pool was labelled with 400 pmol of Cy3 dye per 50 µg of protein, according to manufacturer's protocol. In parallel, equal amounts of proteins from each sample were mixed to form an internal standard (IS). The former was labelled with Cy5 in the same manner. Each labelled sample was combined with a equal amount of the labelled IS (50 µg: 50 µg) and mixed with rehydration buffer for the following final composition: 7 M urea, 2 M thiourea, 2% (w/v) 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulphonate (CHAPS), a trace of bromophenol blue, and 1.2% (v/v) DeStreak reagent. The sample was left to incubate for 30 min at room temperature and centrifuged for 10 min at 14,000 g. Each combined sample was applied to a 24 cm immobilized pH gradient strip (pH 4-7) using cup-loading. Proteins were focused for a total of approximately 90 kV/h, during which the voltage was gradually increased up to 8,000 V for a total of 25 h. After focusing, proteins were reduced and then alkylated for 15 min each step, by soaking immobilized pH gradient (IPG) strips at room temperature in 75 mM tris-HCl, pH 8.8; 6 M urea; 30% (v/v) glycerol; 2% (w/v) sodium dodecyl sulphate (SDS), completed with 1% (w/v) dithiothreitol (DTT) and 4% (w/v) iodoacetamide, respectively. Equilibrated strips were then horizontally applied on top of a 11% SDS- Polyacrylamide gel electrophoresis

(PAGE) gel and proteins were separated vertically at 15°C, using the EttanDaltSix system from GE HealthCare and applying a constant current of 50 mA/gel.

Each gel was scanned at 100 µm resolution using an Amersham Biosciences Typhoon 8,400 variable imager, resulting in two images, one for the IS and one for the sample. Spot detection, gel matching and statistical analysis were performed with Progenesis SameSpots (Non Linear Dynamics). Abundance values of matched spots across all gel images, expressed as normalized volume, were compared between groups, so that each spot could be assigned to a score of relative significant difference, in terms of p-value.

Protein identification

After in-gel digestion of protein spots collected from preparative gels ran with 700 µg of protein stained with Flamingo (BioRad), according to manufacturer's protocol, proteins were identified by Mass Spectrometry (MS) analysis. The spot picking was performed manually on an UV table. In gel-digestion was performed as described before (Bensalem et al. 2007; Shevchenko et al. 1996). In brief, protein spot was washed in milli-Q water, destained in 50% acetonitrile (ACN) and subsequently with 100% ACN. Gel piece was dried before rehydration at 4°C in digestion buffer, containing 50 mM NH_4HCO_3 and 6.7 ng/µL of trypsin. After 30 min, the supernatant was discarded and 10 µL of 50 mM AmBic were added. Digestions were allowed to proceed at 37°C overnight (12-16 hours). Digested peptides present in the supernatants were joined with those extracted from gel pieces before lyophilized to dryness. Tryptic peptides, prepared in 50% (v/v) ACN and 0.1% (v/v) trifluoroacetic acid (TFA), were directly deposited on a 192-well matrix-assisted laser desorption/ionization *(*MALDI) plate with 5 mg/mL α-Cyano-4-hydroxycinnamic acid (α-CHCA) (1:1), prepared in 0.1% TFA/60% ACN (v/v) and allowed to co-crystallize at ambient temperature.

Peptides were analyzed on an Applied Biosystems 4,700 Proteomics Analyzer with Time-of-flight/time-of-flight (TOF/TOF) ion optics. Data were acquired in positive MS reflector mode with six spots of standard (Calibration Mixture 2, Applied Biosystems) used for calibration (4,000 Series Explorer Software v3.0 RC1). MS spectra were obtained by 1,000 shot/sub-spectrum accumulations. Five precursor peaks, with the best signal to noise ratios (S/N), were selected from each spectrum for MS/MS analysis. For MS/MS spectra, a maximum of 5,200 laser shots were

accumulated. Data interpretation was carried out using GPS Explorer software (Version 3.5, Applied Biosystems) and a local copy of the MASCOT search engine (Version 2.0).

For combined MS and MS/MS analysis, all peptide mass values are considered monoisotopic, a MS mass tolerance was set at 50 ppm and a MS/MS fragment tolerance set at 0.25 Da. Trypsin was assigned as the digestion enzyme, a single missed cleavage site was allowed and carboamidomethylation of cysteinyl and oxidation of methionyl residues, were assumed as fixed and variable modifications, respectively. A taxonomic restriction to *Homo sapiens* protein sequences was included. For MS, all peaks with a signal to noise (S/N) greater than 5 and for MS/MS all peaks with a S/N greater than 3 were included in the database search against the Swiss-Prot database (Swiss-Prot 2012.02.10, 534,242 sequences, 20,317 *Homo sapiens* sequences). The criteria used to accept the identification were significant homology scores achieved in Mascot ($p<0.05$). Identified proteins were automatically annotated using Protein information knowledge extractor (PIKE) software.

Descriptive and statistical details

Quantitative data are described by several measures according to their distribution namely mean, standard deviation – SD, median (or 50^{th} percentile), minimum (min.) and maximum (max.), while qualitative data are described in absolute values (and the respective percentage).

Differences between the sampled sites in what concerns $PM_{2.5}$ and PPAH were assessed based on Mann Whitney paired test for each pair of sites (with Holms sequential Bonferroni adjustment of the significance level). Statistical differences between the study groups in TAS and protein expression values were assessed with ANOVA and with Kruskal-walis test for 8-OHdG. Differences in TAS and 8-OHdG between S and NS were assessed with Mann-Whitney U. Data analysis was performed in Excel and Access from Microsoft Office 2010 and statistical analysis was based on two-sided tests with a 0.05 significance level (α) and performed in SPSS version 19.0.

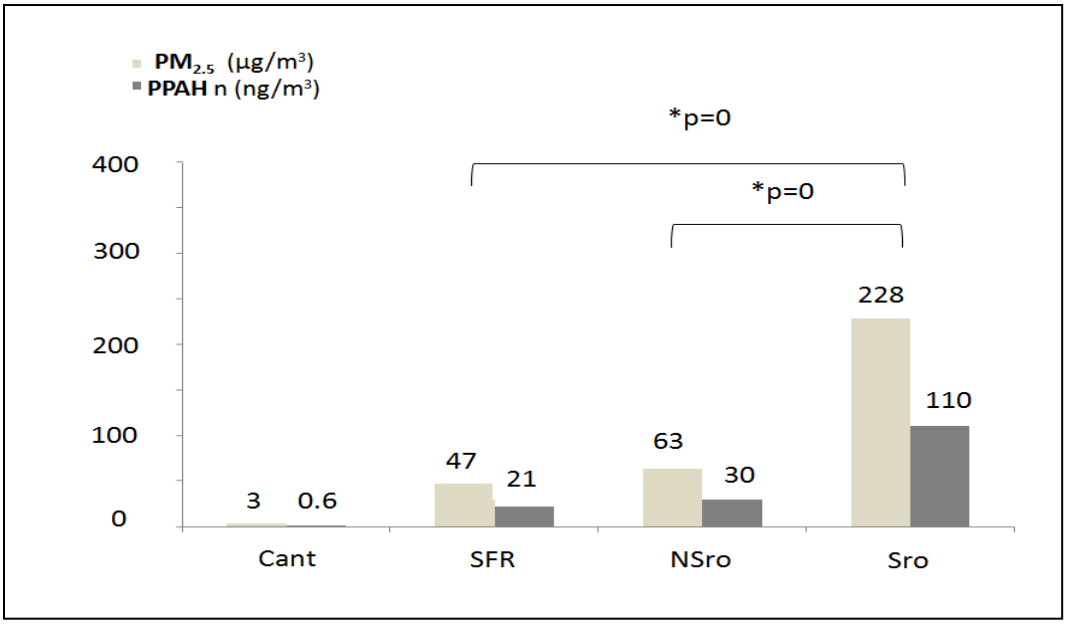
Results

$PM_{2.5}$-to-PPAH levels

In a recent study, published in the peer-reviewed literature, we have measured real-time $PM_{2.5}$ inside and outside 25 restaurants and four canteens located in Lisbon

(Pacheco et al. 2012). Here, we estimated SHS-specific particulate-bounded PAH levels at the monitored locations based on the model published by Repace et al. (2004). Accordingly, the calculated PPAH levels reached 110 ng/m^3 in Sro, a level significantly higher than those calculated for NSro (30 ng/m^3), SFre (22 ng/m^3) and Canteens (0.6 ng/m^3) (Figure 9 and Supplementary information: Table 17).

Figure 9: Fine particulate matter (PM) and particulate polycyclic aromatic hydrocarbons (PPAH) pollution at Lisbon restaurants.



Median concentrations of indoor measured $PM_{2.5}$ and estimated PPAH levels for smoking rooms (Sro), non-smoking rooms (Nsro), smoke-free restaurants (Sfre) and canteens (Cant), were subtracted from the outdoor median level. Statistical analysis was based on Mann Whitney paired test for each pair of sites (Sro, NSro, SFre), with Holms sequential Bonferroni adjustment of the significance level (adjusted α); *adjusted α = 0.017, **adjusted α = 0.025.

Characteristics of the study population

Demographics, smoking habits and life style

The studied population consisted of a total of 96 employees recruited from the monitored venues. Most of these employees were males (75%), working at least 40 h/wk, presenting a mean age of 40 years old. In regard to their smoking status, NS were in greatest number (74%), being homogeneously distributed between SDA or NSDA (Pacheco et al. 2012). Given recruitment constrains, former smokers were included in the non-smokers group, provided that smoking cessation had occurred for more than six

months prior to sample collection. In average, former-smokers quit smoking 14 years ago and were also homogeneously distributed between SDA and NSDA.

Other tobacco consumption details were also collected, for both active and former smokers (Table 7). Mean age at which smoking habit began (age at start) was around 16 years old, finishing between 25 and 31 years old in former smokers (age of cessation). The number of cigarettes consumed per day varied between nine and 20, while the Packed years unit (PYU) estimated for this population was calculated to be between nine and 17. Besides smoking status, the cotinine marker, previously measured on these workers (Pacheco et al. 2012), was able to discriminate non-smoker employees working at SDA and NSDA.

Table 7: Workers' demographic and tobacco consumption details, according with the smoking status and working place. Each variable is represented by mean and, in brackets, the standard deviation.

	Non-smokers				**Smokers**			
	NSDA		SDA		NSDA		SDA	
Nº	38	13*	32	16*	12		14	
Age	42 (14)	45 (10)	36 (11)	38* (12)	41	(12)	32	(9)
Age (started smoking)	17*	(3)	16*	(4)	15	(3)	17	(5)
Years of smoking habit	14*	(11)	9*	(11)	26	(12)	15	(7)
Nº of cigarettes in a day	9*	(7)	15*	(20)	13	(6)	20	(7)
Packed years unit	9*	(9)	12*	(23)	17	(13)	16	(11)
Age of cessation	31*	(10)	25*	(9)				
Years since cessation	15*	(10)	14*	(11)				

NSDA, non-smoking designated area; SDA, smoking designated area. *Former smokers that quit smoking at least six months before sample collection.

These study groups were further characterized to evaluate their exposure to SHS at home or another job and other factors such as lifestyle and diet (Supplementary information: Table 18 and Table 19). Accordingly, we verified that most individuals, regardless of their categorization, are not home-SHS exposed and have no other professional activity where they could be occupationally exposed. Importantly, most of the employees always worked on the hospitality industry. Regarding personal lifestyle, the majority of the workers are sedentary (sports activity). This observation is more pronounced among the smokers than non-smokers, although no distinction could be

made between workers SHS-exposed or not exposed. Usually the workers have three or more daily meals and eat equal amounts of fish and meet in a week. Alcoholic beverages consumption was also evaluated among the study groups. We observed that the majority of the workers, except the smokers exposed to SHS, tend to drink more than one type of alcoholic beverage such as beer, wine and other types and that they usually drink three to four cups a day.

Respiratory function

The majority of enrolled workers was clinically examined to exclude pre-existence of respiratory disorder and evaluated their respiratory function by a spirometry test. From the 78 spirometry tests performed, due to calibration problems, seven did not meet acceptable criteria and were excluded. As summarized in Table 8, and in accordance to GOLD guidelines, all workers registered lung function parameters within the normal reference range: FVC and FEV1 indices above 80% and the ratio of FEV1 to FVC above 75%.

Table 8: Summarized spirometry results per each study group. Relevant demographic data was collected before measurement of the three main spirometry indices (FVC, FEV1 and FEV1/FVC). Each variable is represented by mean and, in brackets, the standard deviation.

Gender	Age	Weight (kg)	Height (cm)	BMI (kg/m^2)	FVC (%)	FEV1 (%)	FEV1/FVC (%)
M (N=18)	44 (13.3)	78 (10.3)	169 (8.4)	27 (2.9)	91 (12.3)	94 (14.6)	81 (4)
F (N=11)	46 (10.6)	70 (8.2)	161 (8.4)	27 (2.7)	96 (8.1)	96 (11.7)	81 (4.6)
M (N=22)	39 (10.7)	77 (12.1)	172 (6.7)	26 (3.9)	91 (8.9)	92 (10.4)	81 (5.7)
F (N=3)	34 (16.2)	72 (7.2)	163 (2.6)	27 (3.6)	110 (12.5)	109 (13.1)	84 (6.7)
M (N=6)	43 (12)	75 (16.4)	168 (8)	26 (4.6)	102 (25.6)	99 (22.2)	76 (5.8)
F (N=4)	34 (11.4)	58 (9.5)	163 (6.6)	22 (3.5)	104 (5.1)	99 (5)	80 (1.3)
M (N=7)	31 (7.2)	80 (10)	174 (6.3)	26 (2.9)	92 (8.2)	95 (11.7)	84 (6.7)
F (N=1)	50	85	172	29	82	83	80

Study groups: non-smokers not exposed (NSNE), non-smokers exposed (NSE), smoker not exposed (SNE) and smoker exposed (SE). Forced vital capacity (FVC), forced expiratory volume in one second (FEV1), ratio of FEV1 to FVC (FEV1 /FVC) and body mass index (BMI).

Analysis of SHS-induced biochemical and molecular changes

8-Oxo-2'-deoxyguanosine

Serum levels of 8-OHdG were determined in 80 samples (Table 9) and no significant differences were observed across the four study groups (Chi-Square=5.09; p=0.17). However, the mean serum level of 8-OHdG was found to be much higher in NSE workers (1.17 ng/ml) when compared with all the other groups. Within the smoker workers, higher levels were also found in the occupationally SHS exposed (0.72 ng/ml). Any statistical difference was found between smokers and non-smokers (Mann-Whitney U=431; p=0.07).

Table 9: Workers' serum levels of 8-hydroxy-2'-deoxyguanosin (8-OHdG).

		NSNE	NSE	SNE	SE
8-OHdG (ng/ml)	N	32	29	11	8
	Mean	0.89	1.17	0.56	0.72
	SD	1.12	0.99	1.14	0.86
	Min.	<0.125			
	Max.	3.6	3.11	3.86	2.09

Study groups: non-smokers not exposed (NSNE), non-smokers exposed (NSE), smoker not exposed (SNE) and smoker exposed (SE).

Total antioxidant status (TAS)

Before proceeding to plasma protein profiling, we investigated its TAS. According to our results (Table10), we found higher levels in SE workers although no significant differences were observed between the studied groups (f=0.143; p=0.93) and between smokers and non-smokers (Mann-Whitney U=242; p=0.82).

Table 10: Total Antioxidant Status (TAS) determined in workers' plasma.

		NSNE	NSE	SNE	SE
TAS (mmol/l)	N	15	13	10	8
	Mean	1.104	1.099	1.068	1.12
	SD	0.196	0.175	0.152	0.178
	Min.	0.76	0.76	0.7	0.75
	Max.	1.38	1.38	1.27	1.31

Study groups: non-smokers not exposed (NSNE), non-smokers exposed (NSE), smoker not exposed (SNE) and smoker exposed (SE).

Plasma protein analysis

As mentioned, plasma proteome analysis was based on a strategy of 2D-DIGE MS using MALDI-TOF/TOF instrumentation. Briefly, 2D-DIGE separation approach resulted in twelve 2D-separated maps, as illustrated in Figure 10. Each 2-D maps was scanned into a 16-bit image and then aligned for spot matching. Altogether, about 1000 protein spots were separated on each gel. The abundance level of these matched spots across all gel images was then compared between the study groups. In total, 61 spots exhibited an abundance variation according to a fold change equal or higher than 1.3 given a $p<0.05$. The rational for using this criteria is that small changes may have large biological effects, as several studies had demonstrated (Bortner et al., 2011; Hinnebusch, Meng, Wu, Archer, & Hodin, 2002). Out of the 61 spots, we were able to excise 32 from a preparative gel, from which 21 were identified through MS. These spots corresponded to nine different proteins. Functional annotation showed that these proteins are involved in metabolism, acute-phase inflammation, transport, immune and vascular function. Table 11 describes protein spots that exhibited abundance changes between NSE workers and unexposed ones (NSNE). Each protein spot is represented according to its numeration, as showed in Figure 10, and is attached to the correspondent MS details.

Figure 10: 2-DE reference map of plasma depleted from the 14 most abundant proteins. Differentially expressed spots are numbered and pointed with arrows.

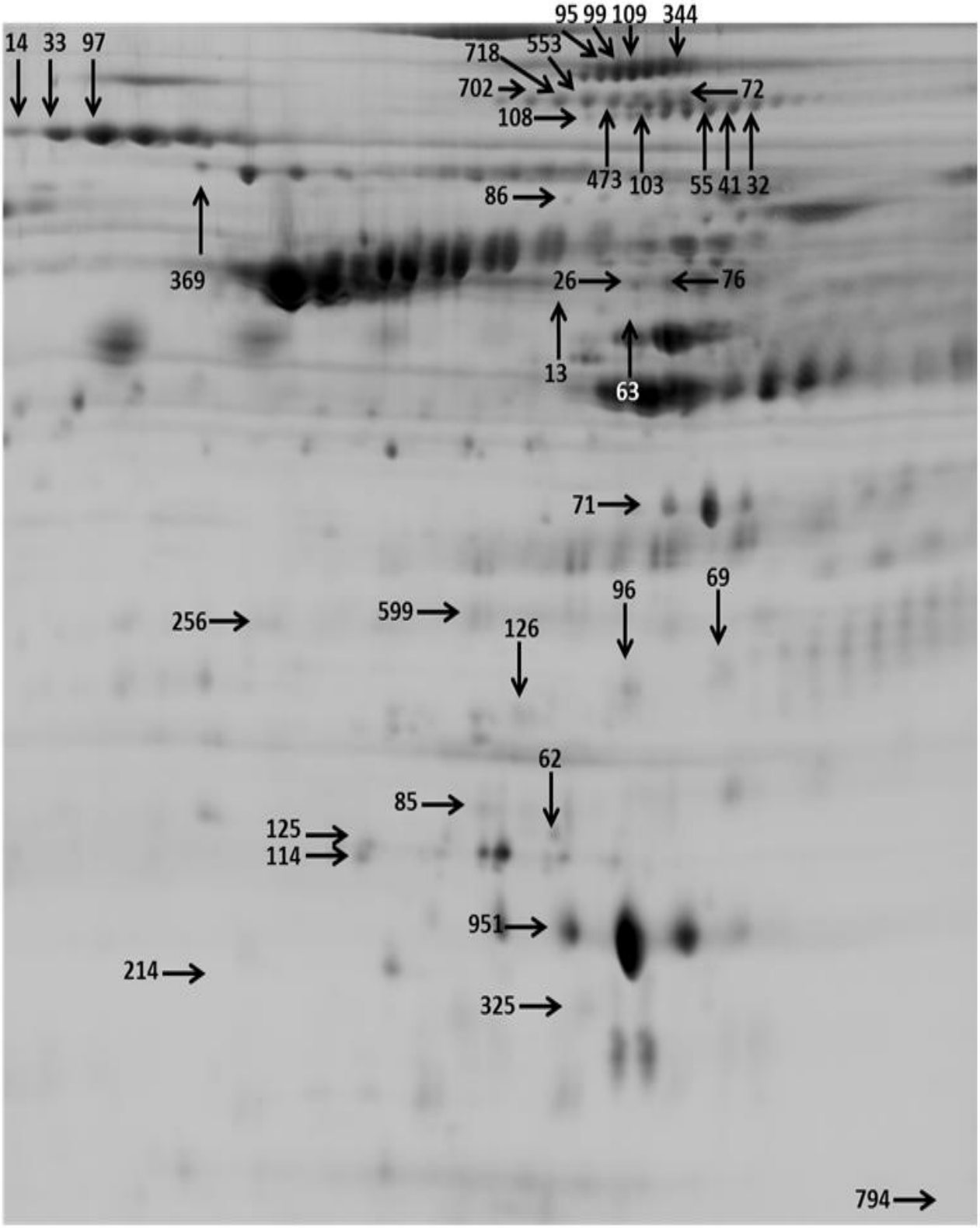


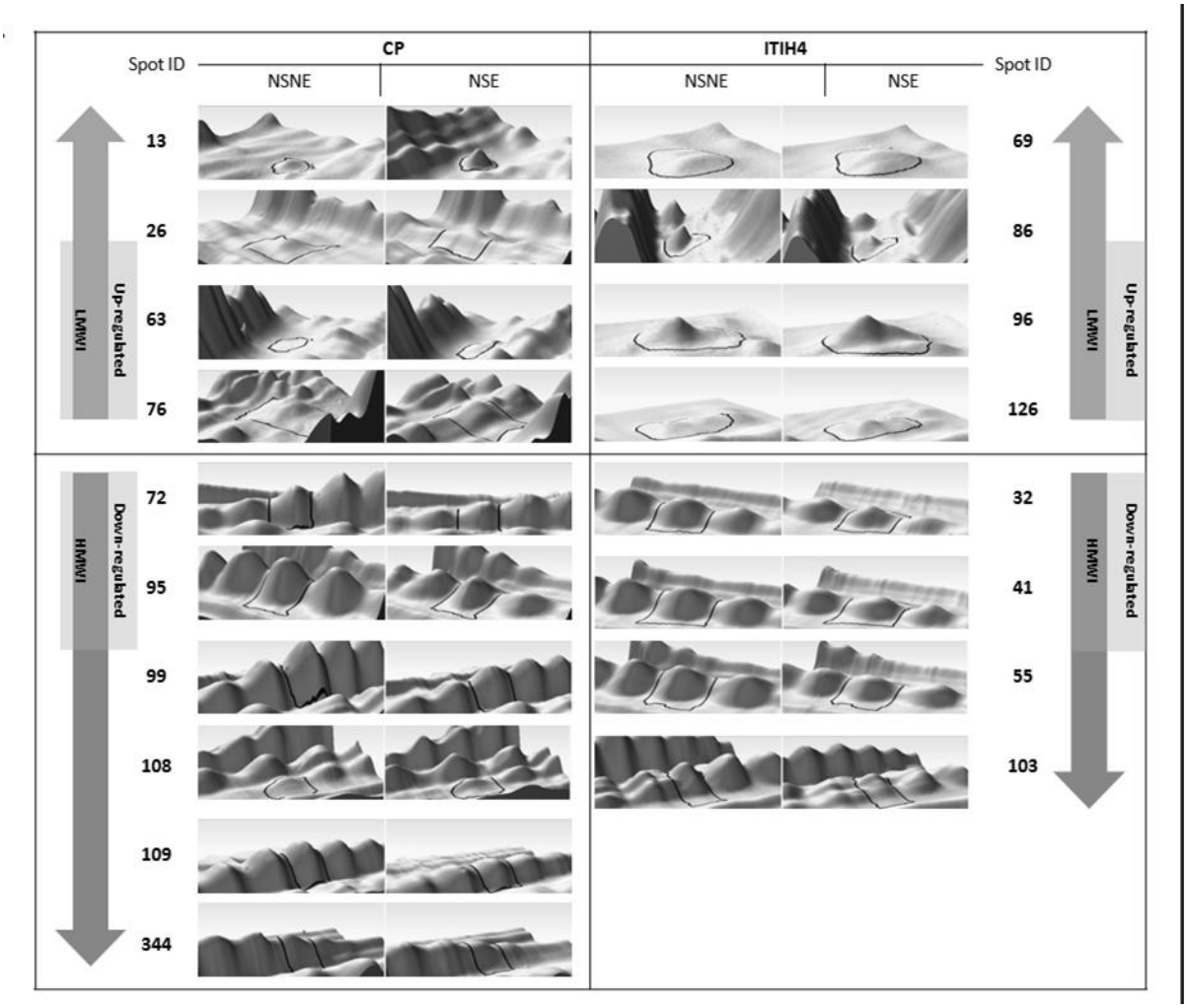
Table 11: Data from the SameSpots analysis (Spot ID, Fold Change and p value), together with relevant MS data for the 21 differentially expressed identified protein spots.

Protein name (UniProt ID)	spot ID	Fold change	p value	Mascot Score	Sequence coverage	Queries matched	Sequenced ions
Ceruloplasmin (P00450)	13	2.5	0.01	318	11	20	6
	26	2.1	0	339	12	19	6
	63	1.7	0.01	224	10	15	5
	76	1.6	0.01	374	16	23	6
	72	-1.6	0	254	13	18	4
	95	-1.4	0.01	626	25	35	9
	99	-1.4	0.03	583	26	36	10
	108	-1.4	0.03	69	9	11	3
	109	-1.4	0.05	547	26	35	9
	344	-1.4	0.05	587	32	33	6
Inter-alpha-trypsin inhibitor heavy chain 4 (Q14624)	69	1.6	0	132	8	14	4
	86	1.5	0	95	9	12	4
	96	1.4	0.01	88	6	9	3
	126	1.3	0.02	123	7	8	2
	32	-2	0	114	19	19	1
	41	-1.9	0	544	29	35	6
	55	-1.8	0	313	25	29	5
	103	-1.4	0.01	354	24	28	3
Serum amyloid P-component (P02743)	85	1.5	0.02	453	34	18	6
	62	-1.7	0.03	61	20	6	2
	108	-1.4	0.03	91	9	22	4
	114	-1.3	0.00	429	33	17	6
	125	-1.3	0.01	241	33	13	4
Apolipoprotein A-IV (P06727)	71	-1.6	0.01	895	70	44	11
Apolipoprotein M (O95445)	325	1.4	0.05	294	37	17	5
	256	1.5	0.06	349	40	18	7
Complement factor B (P00751)	14	-2.5	0.01	549	39	36	7
	33	-2	0.01	229	20	17	6
	97	-1.4	0.02	757	41	37	9
Ficolin-3 (O75636)	214	-1.7	0.05	101	10	5	2
Gelsolin (P06396)	369	-1.3	0.19	610	35	36	8
Antithrombin-III (P01008)	63	1.7	0.01	216	24	8	2

Note: Positive fold change means increased expression level, or up regulation of the isoform in non-smoker workers exposed versus not-exposed (NSE vs NSNE respectively)

Among these group of proteins spots, more than a half corresponded to Ceruloplasmin (CP, n=10) and Inter-alpha-trypsin inhibitor heavy chain 4 (ITIH4, n=8), revealing the existence of several isoforms of these proteins. Examining in detail the pattern of CP and ITIH4 (Figure 11) we observed that all down-regulated spots in exposed workers corresponded to their high-molecular-weight isoforms (HMWI). In parallel, we observed that low-molecular-weight isoforms (LMWI) were increased in exposed workers.

Figure 11: 3-D view of Ceruloplasmin (CP) and Inter-alpha-trypsin inhibitor heavy chain 4 (ITIH4) spots according to the condition studied: SHS-exposed workers (NSE) and controls (NSNE).



Arrows pointing up and down stand for over and under expression on exposed workers respectively. The amount of the protein isoform is proportional to the volume of the peak, which is graphically represented here. The volume of each spot was calculated considering the total pixel value per spot area, subtracted by the correspondent background. Down-regulated and up-regulated protein isoforms of CP and ITIH4 are represented by the same numeration used in table 10, and correspond to negative or positive fold change respectively. LMWI (low-molecular-weight isoform), HMWI (high-molecular-weight isoform)

Discussion

In the current study, our overall goal was identifying biomarkers that could link SHS occupational exposure and the onset of smoking-related diseases. In a previous study, where we focused on indoor SHS air pollution, we provided evidences that this contaminant remains high in restaurants where smoking is allowed, regardless of the protective measures used, such as ventilation systems. This observation was essentially based on monitoring indoor $PM_{2.5}$, a marker that registered high levels on those places and that we found to be strongly positive correlated with the number of smoker occupants in the smoking rooms. In the current work, we have estimated the levels of PAHs that are known to be associated with the particulate phase of SHS. Repace and coworkers (2004) published a model demonstrating that the ratio of fine particles to PPAH in SHS is about 2:1 (when these parameters were expressed in units of micrograms per cubic meter and nanogram per cubic meter, respectively) and that each parameter strongly correlates with smoker density (Repace et al. 2006; Repace et al. 2011). Based on Repace's previous model (2004), we estimated that the levels of the PPAH in smoking rooms could be as high as 110 ng/m^3, a value significantly higher than that estimated for non-smoking rooms (30 ng/m^3). Seeking on published literature, we found these values between the ranges of concentrations measured on smoking casinos (Jiang et al., 2011; J. Repace, 2004; York & Lee, 2010).

How the amount of tobacco smoke-specific PPAHs is associated with an increased risk of an acute and chronic respiratory and cardiovascular effect is not quantified. These chemicals could have multiple sources of emission and their abundance could present some variation, making impossible to define an absolute safe level of exposure. Benzo[a]pyrene (B[a]P) is one of the components of tobacco smoke and is probably the best known PPAH, classified by IARC as an human carcinogen (IARC, 2010). Besides cancer, several other adverse health outcomes have been associated with the exposure to B[a]P, namely birth defects, genetic damage, immunodeficiency, respiratory and nervous system disorders (Patil et al., 2009; Petruzzelli et al., 1998). Therefore, B[a]P has been used as a marker of PAH mixtures, irrespective of the environment (Delgado-Saborit, Aquilina, Meddings, Baker, & Harrison, 2011). According to WHO guidelines, the concentration for lifetime exposure to indoor air B[a]P producing excess lifetime cancer risks of 1/10,000 is approximately

1.2 ng/m^3 (WHO, 2010). In the present study, although the estimated level of PPAHs corresponds to the sum of all individual PPAHs, one might expect that employees working in smoking rooms could be at higher risk of developing cancer than those not exposed (assuming that other sources of carcinogens have the same weight between individuals in this population). Our measurements of cotinine in workers urine in the end of their shift, at the same day we have monitored SHS air pollution, reinforce this idea. In fact, we collected evidences showing that workers of smoking rooms are indeed exposed to SHS, inhaling its hazard compounds (Pacheco et al. 2012).

Besides worker's occupational exposure and smoking habits, we evaluated other relevant factors such as the exposure to SHS at home or another job or even the exposure to another source of fine particles and PPAH, namely the use of fireplace at home. From the analysis of questionnaires, we didn't find any significant difference between the four study groups. Importantly, it was stressed out that the majority of these individuals always worked on the hospitality industry. Thus, it is expectable that the most important local of exposure to SHS is the workplace in study. Workers were also evaluated for respiratory symptoms (results not shown) and submitted to spirometry test to evaluate their lung function. In our population, namely non-smoker employees, the three spirometric indices FVC, FEV1 and FEV1 /FVC couldn't discriminate whether if they work on SDA or NSDA. All workers presented a normal lung function, including smokers. The absence of any relationship between lung function and levels of exposure to tobacco smoke could be due to the fact this population is relatively young, aging in average 40 years old and are not considered heavy smokers.

To fill the gap between occupational SHS exposure and the onset of SHS-associated diseases, we searched for biochemical and molecular differences between workers exposed and unexposed to SHS. TAS and 8-OHdG levels, two parameters of oxidative stress, were measured on workers' plasma and serum, respectively, and none were sensitive enough to detect significant differences. However, SHS-exposed employees exhibited higher mean levels of 8-OHdG than those not exposed, regardless of their smoking status. In a previous study, Howard and co-workers analyzed several oxidative markers in a non-smoker employee population, finding that exposure to SHS resulted in a statistically significant increase of 8-OHdG levels in the DNA extracted from blood leucocytes (Howard et al., 1998). Interestingly, our collaborators

investigating the effects of SHS on DNA damage also couldn't find differences between the recruited workers, whether they were exposed or not to SHS. This study was based on the assessment of DNA breaks in blood leukocytes using the comet assay. However, significant differences were observed regarding DNA repair capacity. This was evaluated by challenging blood cells with the mutagenic drug Ethyl methane sulfonate (EMS) before proceeding with the comet assay, revealing a significantly lower level of DNA breaks in SHS-exposed employees as compared to non-exposed workers, regardless of their smoking status (Louro, 2011). This observation suggests that SHS, possibly at low levels, might activate a responsive mechanism that counteracts the negative effects SHS could exert on DNA level.

These results prompt us to investigate if there were differences between study groups at protein level. Possible protein changes induced by SHS were searched on depleted plasma using a proteomics approach, named 2D-DIGE coupled with mass spectrometry. While plasma depletion facilitated the observation of medium to low level expressed plasma proteins, 2D-DIGE separation approach provided an accurate method to compare and detect protein profiles differences associated to each study group, namely those corresponding to SHS exposed workers compared with unexposed ones. Among the proteins that exhibited alteration of their abundance, CP and ITIH4 became prominent. These two proteins presented a high number of isoforms and suffered the highest abundance variation induced by SHS. Importantly, either this variance consisted on an increase or decrease of isoform abundance, this behaviour was associated with the exposure to SHS, regardless of the smoking habit of the exposed workers. A detailed observation of the CP and ITIH4 isoforms, highlighted the fact all HMWI suffered a decrease in their abundance and, in parallel, LMWI suffered an increase in their abundance. This expression patterns suggests that SHS might induce on CP and ITIH4 a specific proteolytic cleavage or an increased instability, possibly due to oxidative modifications. Interestingly, CP and ITIH4 are acute-phase inflammation proteins that were already referred on literature as exhibiting abundance variation. In some of these studies, CP and ITIH4 fragmentation are associated with a health dysfunction (Abdullah-Soheimi, Lim, Hashim, & Shuib, 2010; Kim et al., 2011; Squitti, Quattrocchi, Salustri, & Rossini, 2008; Tewari, Popova-Butler, El-Mahdy, & Zweier, 2011). The exact pattern of fragmentation induced by SHS remains to be investigated.

Considering that variation levels of CP and ITIH4 isoform were dependent on SHS exposure, whether the workers were smokers or non-smokers, following a similar pattern observed for the 8-OHdG maker and the DNA repair assays, it seems that SHS affects not only non-smoker employees but also active smokers at this leisure establishments.

Conclusion

This study aimed at examining the possible biochemical and molecular effects of SHS on occupationally exposed workers, preceding the onset of the first symptoms of chronic disease, namely the decline of lung function. Firstly, we have demonstrated that partial smoking restrictions in Portuguese leisure establishments provided no full protection to their employees, whatever protective measures are used (Pacheco et al. 2012), supporting the need for smoke-free laws at these places. Here, despite the known difficulties to biomonitor the exposure to SHS, we were able to detect significant protein changes, namely on CP and ITIH4, that might constitute biomarkers of the effects that SHS exerts on exposed workers of the hospitality industry.

3rd study

Early predictors of secondhand smoke exposure in the plasma proteome

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Abstract

Every year, more than 600 thousand non-smokers dye due to secondhand smoke (SHS) exposure. Children account for a noteworthy part of the deaths. Additionally, for many non-smokers, SHS in the workplace has been linked to an increased risk for heart disease and lung cancer.

In 2007, while several countries moved to a total tobacco ban, Portugal implemented a partial smoking restriction in public venues. The evaluation of a group of restaurants in Lisbon, revealed that the majority of smoker rooms of these restaurants were still highly contaminated with SHS, despite using reinforced ventilation systems. The workers from this smoking rooms, presented higher urine levels of cotinine, a metabolite of nicotine and, most interesting, significant DNA and protein changes in their blood (Pacheco et al 2012; Pacheco et al 2013).

Considering that these molecular events may precede the onset of the first symptoms of tobacco-related diseases, in the present study we aimed to apply a

complementary proteomic approach to support previous results, towards the identification of SHS exposure candidate biomarkers, in the plasma of occupationally exposed workers.

Together with previous results, a panel of four proteins were prominent, namely ceruloplasmin (CP), inter-alpha trypsin inhibitor Heavy chain 4 (ITIH4), gelsolin (GSN) and alpha-1-acid glycoprotein 2 (ORM2). All have been related with cancer and other conditions that are frequent outcomes of tobacco smoke exposure. This suggests they might be critical in SHS exposure response. Therefore they should be used for future prevention on behalf of occupational, or more broadly, public health.

Introduction

The global tobacco epidemic is just starting, due to the decade(s) interval between the start of the exposure and the health outcomes. Still, tobacco is already one of the biggest public health threats. In the last century, it caused 100 million deaths and may cause one billion deaths in the 21st century (WHO, 2013).

When people burn tobacco products, secondhand smoke (SHS) spreads and accumulates in enclosed spaces, such as homes, public venues and offices. Almost half of children regularly breathe air polluted by tobacco smoke in public places, and over 40% have at least one smoking parent. In 2004, children accounted for 28% of all deaths related with SHS exposure (WHO, 2012c).

Near 70, out of the 250 hazardous chemicals in tobacco smoke, are known to cause cancer and there is no safe level of exposure to SHS. In adults, SHS exposure causes cardiovascular and respiratory diseases, such as lung cancer and coronary heart disease (NIOSH, 2015).

Surveillance together with scientific research, are key to track the extent and character of the tobacco epidemic, and indicate how best to tailor public health and welfare policies (Bach, 2007).

In 2007, several countries had already smoke-free public venues. Meanwhile, in Portugal, a partial smoking restriction was implemented provided that several protection measures against SHS exposure were in place (Law 37, 2007). The scarcity of information on the impact of this law triggered this project, aiming to understand if these venues workers are effectively protected from SHS exposure.

The first results revealed that the smoking areas in these venues were still contaminated with tobacco smoke. Also, the protective measures adopted did not prevent workers from inhaling polluted air (Pacheco et al., 2012). All workers exhibited a normal lung function and no significant differences were observed in the blood levels of two oxidative markers. Conversely, a preliminary proteomic study, by two-dimensional difference gel electrophoresis, coupled with a matrix-assisted laser desorption/ ionization tandem time-of-flight (2D-DIGE-MALDI-TOF/TOF), identified nine differentially expressed proteins, in pooled samples of depleted plasma. Out of these, two acute-phase inflammation proteins, ceruloplasmin (CP) and inter-α-trypsin inhibitor heavy chain H4 (ITIH4), presented a high number of isoforms, and a similar expression pattern, in SHS exposed workers (Pacheco et al., 2013).

Despite all the efforts it is still unclear to medical and scientific community how non-smokers develop chronic diseases induced by SHS exposure. Therefore, we believe that unraveling these molecular mechanisms is crucial to understand, and hence prevent, SHS related illness.

Accordingly, to complement and corroborate preliminary results, we now aim to identify a larger panel of candidate biomarkers, in blood plasma of never-smoker subjects, that are occupationally exposed to SHS and further validate a protein subset.

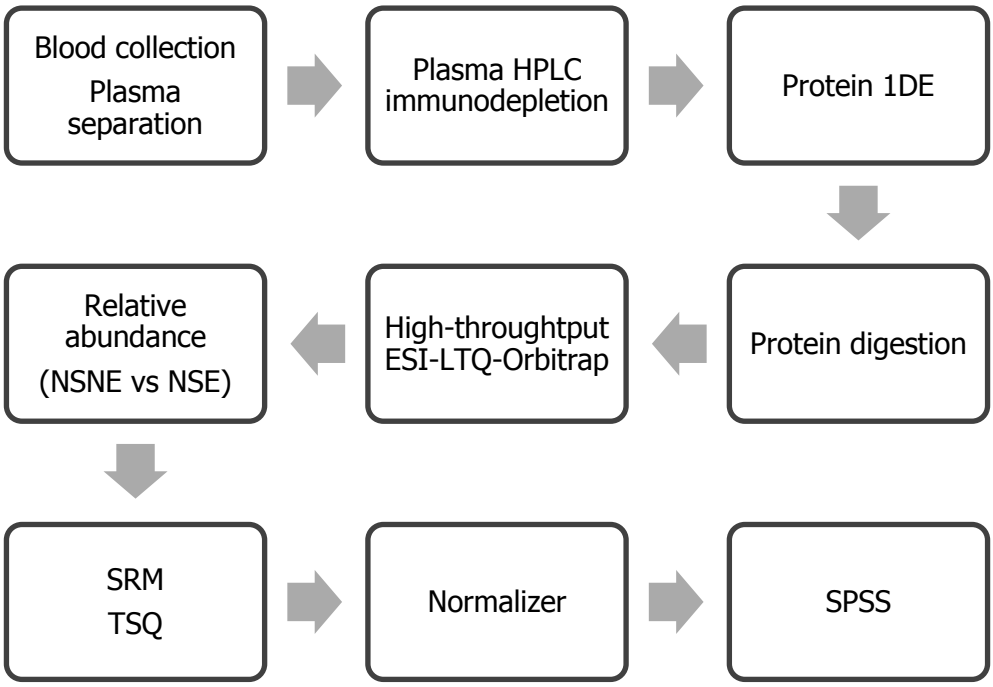
Material and Methods

Glycine, Tris(hydroxymethyl)aminomethane (Tris Base) purchased from GE Healthcare (Uppsala, Sweden). Dithiotreitol (DTT), trifluoroacetic acid (TFA) and protease inhnibitor (PI) purchased from Sigma Aldrich (Stockholm, Sweden). Acetonitrile (ACN) and ammonium bicarbonate (AmBic) purchased from Fluka (Sigma Aldrich, Stockholm, Sweden). Formic acid (FA) purchased from J.T. Backer VWR (Sigma Aldrich, Stockholm, Sweden). Laemmli sample buffer, 12% Criterion TGX Gel, 12+2 well, 45 µl and Precision Plus Protein Standard purchased from Bio-Rad (Hercules, CA). Sequencing grade modified trypsin purchased from Promega Biotech AB (Nacka, Sweden). BCA protein assay kit purchased from Pierce Chemical Company (Nordic Biolab,Stochholm Sweden). Iodoacetamide (IAM) purchased from AppliChem and sodium chloride (NaCl) purchased from Honeywell (Saveen Werner, Malmö Sweden). GelCode blue stain purchased from Nordic Biolab (Stochholm Sweden).

Acetonitrile (ACN) and water for HPLC purchased from Merck (Solna, Sweden). S-monovette ethylenediamine tetraacetic acid (EDTA) KE/7.5 mL tubes from Sarstedt (Nümbrecht , Germany). The Multiple affinity removal system (MARS) Human 14 (4.6 mm id × 50 mm), the Buffers A and B, molecular weight cut-offs (MWCO) (Amicon Ultra 4, Millipore) and the a 0.22 µm spin filter, obtained from Agilent Technologies, Inc. (Wilmington, DE). Spin SpikeTides peptide technologies GmBH acquired from JPT Peptide Technology (Berlin, Germany).

The study workflow is depicted in Figure 12.

Figure 12: Workflow.



Blood collection, plasma separation and depletion

Thirty four workers provided blood samples that were processed in plasma and serum as described elsewhere (Charro et al. 2011), before storage at -80ºC. In order to enhance the detection of lower abundant proteins, the plasma was depleted from the 14 most abundant proteins (albumin, Immunoglobulin A, M and G, α-1-antitrypsin, transferrin, haptoglobin, fibrinogen, α-2-macroglobulin, α-1-acid glycoprotein, apolipoprotein AI and AII, complement C3 and transthyretin), according to manufacturer's instructions. Briefly, before injection onto a MARS column, the human

plasma was diluted 4X with Buffer A, filtered with a 0.22 µm spin filter and centrifuged for 1 min at 16,000 g to remove particulates. Individual plasma samples were depleted and un-bound fractions were concentrated and buffer-exchanged to 25 mM of AmBic by centrifugal filtration using 5 kDa MWCO spin concentrators. Protein concentrations were established using a BCA protein assay kit. Samples were stored at −80°C until further analysis.

Protein electrophoresis, digestion and peptide extraction

Twenty microliters of laemmli sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS and 0.01% Bromophenol Blue together with 350 mM DTT were added to each sample. After vortex and quick centrifugation (16,000 g, 20 sec at 4°C), samples were heated at 95°C during 5 min and then proteins migrated until they were into one single lane, in 12% precast gels (applying 300 V constant current, for 3 min at RT). Later the gels were washed three times, stained using the GelCode Blue Stain Reagent and each lane was cut manually in 9 squares of $1mm^2$. The slices were distained (in 50% ACN and 25 mM AmBic, during 30 min at RT, repeated twice), before performing protein reduction (using 10 mM DTT in 100 mM AmBic) during 1h at 57°C. Protein alkylation was performed by incubating slices in 100 mM AmBic and 55 mM IAA (for 45 min at RT, in the dark). Slices were then washed twice with 100 µL of, in sequence, 100 mM AmBic followed by ACN 100%; speed-vac and incubated (for 12 h at 37°C) with 150 µl trypsin (12.5 ng/µl in 50 mM AmBic). The peptides were extracted from the gel slices by incubation in 200 µL of 75% ACN and 5% TFA during 30 min at RT, under vortexing, twice. To enhance peptide extraction, 250 µL of 100% ACN were added before speed vacuum and storage at -80. Samples were then resuspended for each LC-MS/MS and LC-SRM runs.

Liquid Chromatography and Mass Spectrometry - shotgun data analysis

To allow an efficient LC-MS/MS analysis samples were resuspended in 0,1% FA (for 30 min) and subjected to C18 spin column-based cleaning procedure. An ESI-LTQ-Orbitrap XL mass spectrometer (Thermo Fisher, Bremen, Germany) interfaced with an Eksigent nanoLC 2DTM plus HPLC system (Eksigent technologies, Dublin, CA, USA), was used for shotgun analysis. The auto-sampler injected 5 µL (a total of 6.67 µg of the protein digests). A blank LC-MS/MS run was used between each 5 analyzed samples.

Peptides were loaded, trapped and washed with a constant flow rate of 10 µl/min with solvent A (0.1% FA), for 15 min onto a pre-column (PepMap 100, C18, 5 µm, 5 mm x 0.3 mm, LC Packings, Amsterdam, Netherlands). The peptides were subsequently separated on a 10 µm fused silica emitter, 75 µm x 16 cm (PicoTipTM Emitter, New Objective, Inc.Woburn, MA, USA), packed in-house with Reprosil-Pur C18-AQ resin (3 µm Dr. Maisch, GmbH, Germany). Peptides were eluted with a flow rate of 300 nl/min with a 60 min linear gradient of 3 to 35% (v/v) ACN in water, containing 0.1% (v/v) FA and then increased rapidly to 90% (until min 68) and was kept until min 78, before equilibrating. The instrument was operated in data-dependent mode (DDM) to automatically switch between Orbitrap-MS (from m/z 400 to 2,000 Da) and LTQ-MS/MS (50 to 2,000 Da) acquisition. Mass spectra of all samples were always acquired in the positive ionization mode. Four MS/MS spectra were acquired in the linear ion trap per each Fourier Transform-MS (FT-MS) scan, which was acquired at 60,000 full width at half maximum (FWHM) nominal resolution settings using the lock mass option (m/z 445.120025) for internal calibration. The dynamic exclusion list was restricted to 500 entries, using a repeat count of two, with a repeat duration of 20 sec and with a dynamic exclusion range of two min after the second peptide count. Precursor ion charge state screening was enabled to select ions with at least two charges and rejecting ions with undetermined charge state. The CID energy was set to 35%, and one micro scan was acquired for each spectrum. The complete study was run using five days of MS-instrumentation time. A total of 34 LC-MS/MS runs were performed.

LC-MS/MS raw data was analyzed in Progenesis software, version 2.10.0, which performs automated quantification of peptide abundance. All runs were assessed for the alignment process, but 5 were excluded from peak picking. Features were filtered out when outside RT window of 17-72, m/z higher than 1,600 Da and charge higher than plus five. To correct for experimental variation, and ensure that up- and down-regulated has the same weight, data was normalized. A total of 537,737 MS/MS spectra were searched, against the UniProt human database, including a decoy data set, using Mascot search (Matrix Science). In the search parameters, the enzyme was set to trypsin, with two miss cleavages allowed and peptide and fragment mass tolerances of ten ppm and 0.6 Da, respectively. Carbamidomethylation of Cysteine and the oxidation of Methionine were set as a fixed and variable modifications respectively, and a 0.01

false discovery rate (FDR) threshold was selected at both protein and peptide level. After filtering out "random sequence" proteins, the intensity of only non-conflicting peptides were combined and similar proteins were grouped.

Specific Reaction Monitoring - SRM

An SRM assay was developed for targeted selection and quantification of proteins of interest. UniProt accession numbers were pasted in the SRM management software Skyline, version 2.6, and peptides of 7-25 amino acids length were selected (MacLean et al., 2010). These were checked for uniqueness against a background with the entire human proteome (UniProt KB download). Synthetic peptides, were used to validate the SRM data. The peptides were solubilized according to manufacturer's instructions and a pool of peptides, with a final concentration of 250 fmol/µL, was constructed. The synthetic peptides were run alone and spiked into the plasma samples (8 µL plasma sample (with 6.67 µg) spiked with two µL of the spike tides) and the data was used to validate the peak selection in the analytical samples.

An Eksigent 2D NanoLC system (Eksigent technologies, Dublin, CA, USA) connected to a Triple Stage Quadrupole Mass Spectrometer (TSQ Vantage, San José, CA), with a nano electrospray interface, was used for LC-SRM analysis. Solvents used were mobile phase A (mobA) and B (mobB), which is water/0.1% FA (v/v) and ACN/0.1% FA (v/v) respectively. The LC system was equipped with a trap column (0.3 mm id, 5 mm, PepMap Acclaim C18, LCPackings, Sunnyvale, CA) and in-house packed analytical columns, in 0.075 mm id, 100 mm PicoFrits (New Objective, Woburn, MA), with 3 µm ReproSil C18-AQ particles (Dr. Maisch, Germany) to a length of 12 cm. A 300 nl/min linear LC gradient was delivered starting at 97% mobA and decreasing to 85% mobA during 6 min and then further down to 65% mobA during 68 min. An injection for wash and equilibration of the column was performed between each sample. To control the technical variation, three technical replicates of a pooled sample ran in the beginning, middle and in the end of the SRM experiment.

The TSQ operated in the positive ion mode with a spray voltage of 1.8 kV and an ion capillary temperature of 270°C. A 0.7 Da unit resolution for both Q1 and Q3 were set and a 10 ms dwell time per transition.

Data were acquired using the Xcalibur software, version 2.0.7, and the raw files from SRM data were imported to Skyline to be analyzed and manually inspected. Peak areas for the measured SRM transitions for each individual peptide were integrated and summed to generate the peptide peak areas. Data was exported to Normalizer (Chawade, Alexandersson, & Levander, 2014) and then imported to IBM SPSS statistics, version 22.

The average and median, as well as two-sided Student's t test and Mann Whitney U test, were used whenever data was normally and non-normally distributed, respectively.

Results

The study included 34 workers, mostly males, with ages about 40 years, from 25 public venues (Table 12). None of them reported to have ever smoked. The workers were separated in two groups according with their occupational exposure to SHS. Overall there were 18 subjects not exposed to SHS and 14 exposed to SHS nearly 40 h a week during the last seven years.

Table 12: Workers mean age, body mass index (BMI) and time spent at the workplace (hours and years). The subjects were divided in two groups, according with occupational exposure to SHS.

Never Smokers	**Males**	**Age**	**BMI**	**Workplace**	
				Hours in a day	**Years**
Not exposed to SHS (N=18)	13	45	28	45	5
Exposed to SHS (N=14)	13	34	26	40	7

For the high throughput shotgun analysis, each sample of depleted plasma was analyzed by ESI-LTQ-Orbitrap. In total, 521 proteins were identified, including 11, identified by at least two peptides, that were differentially expressed (t-test, $p \leq 0.05$) and with absolute fold change values - $f \geq 1.26$ (Table 13).

Table 13: Proteins that significantly differentiate never-smokers that are occupationally exposed to SHS.

Accession number	Gene name	p value	fold change	Protein description
P06396-1	GSN	0.003	-1.80	Isoform 1 of Gelsolin
B2RAL6	na	0.010	-1.63	cDNA, FLJ94991, highly similar to Homo sapiens integrin, alpha L (antigen CD11A (p180), lymphocyte function-associated antigen 1; alpha polypeptide) (ITGAL), mRNA
F8WDB8	SYNPR	0.020	-1.52	Synaptoporin
P36980-1	CFHR2	0.040	-1.32	Isoform Long of Complement factor H-related protein 2
B2RA39	na	0.050	1.26	cDNA, FLJ94686, highly similar to Homo sapiens complement factor H-related 5 (CFHL5), mRNA
E9PGP2	F11	0.030	1.28	Coagulation factor XI
Q562E7-1	WDR81	0.020	1.30	Isoform 1 of WD repeat-containing protein 81
P18206-1	VCL	0.020	1.30	Isoform 2 of Vinculin
P12259	F5	0.001	1.30	Coagulation factor V
P05154	SERPINA5	0.003	1.47	Plasma serine protease inhibitor
P19652	ORM2	0.001	1.60	Alpha-1-acid glycoprotein 2

In order to support these results, an SRM analysis was performed for the two manually annotated and reviewed proteins with f≥1.5. All peptides were selected on the basis of their uniqueness to the respective protein, and protein isoforms, and hence, peptides shared with different proteins were excluded from the analysis. The final SRM assay included seven unique peptides, five for GSN and two for ORM2, and 22 transitions (Supplementary information: Table 20).

After total intensity normalization, the peptide integrated peak areas were compared between SHS exposed and unexposed workers and two were confirmed to be significantly different, EVQGFESATFLGYFK, from GSN (p=0.045) and WFYIASAFR, from ORM2 (p=0.021). Both were higher in SHS exposed workers.

Altogether both ORM2 peptides (WFYIASAFR and TEDTIFLR) and GSN peptides EPGLQIWR and EVQGFESATFLGYFK, were increased in SHS exposed workers (figures 13 and 14). Conversely, the three remaining GSN peptides (HVVPNEVVVQR, SEDCFILDHGK and LFACSNK) were decreased in exposed workers (Figure 14).

Figure 13: Box plots comparing ORM2 peptide integrated peaks, between never smoker workers occupationally exposed to SHS (NSE) and not exposed (NSNE).

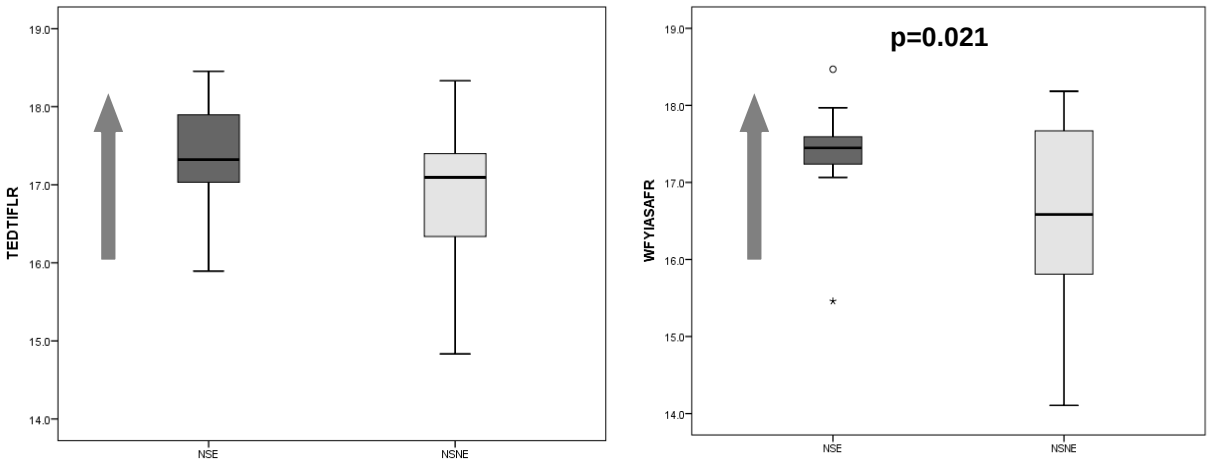
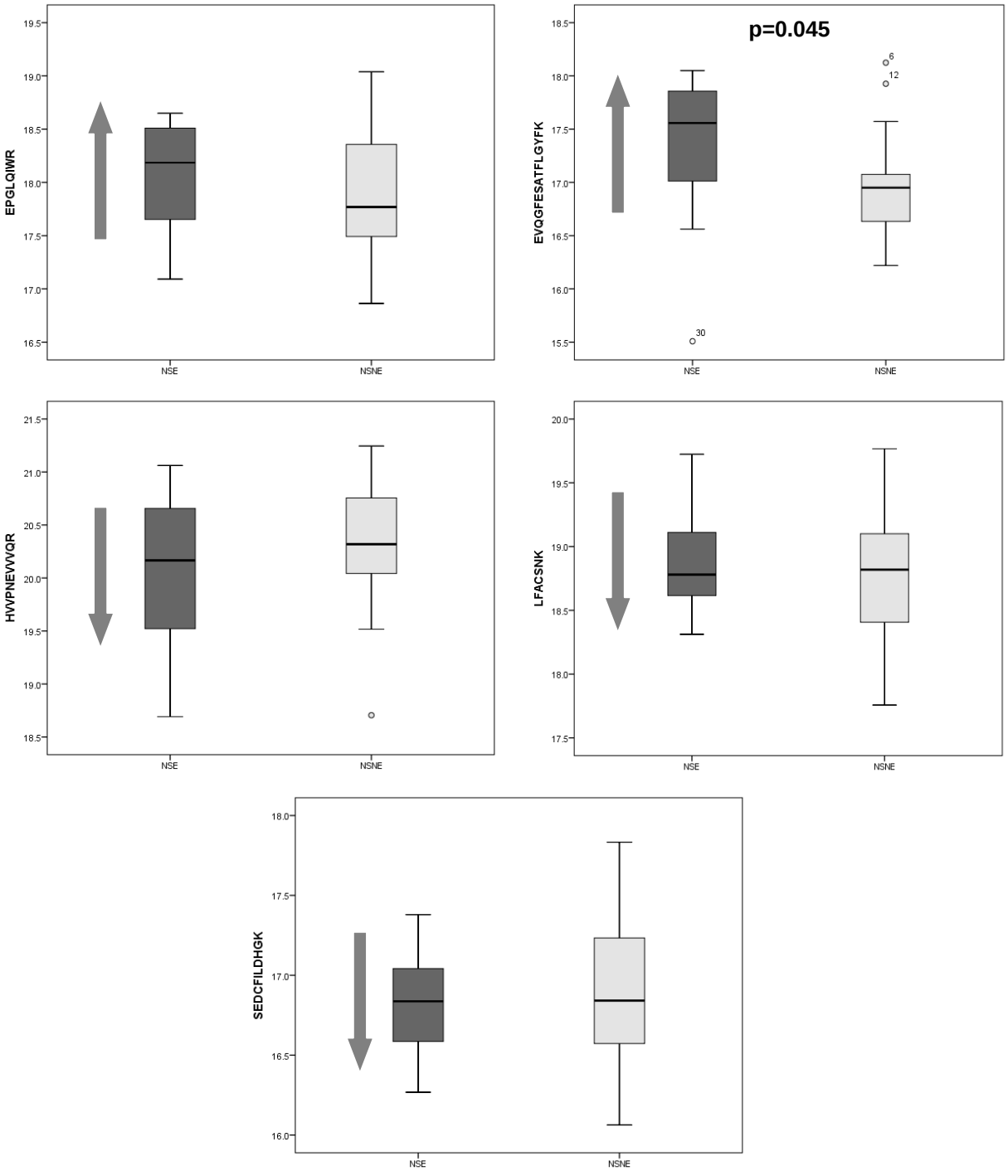


Figure 14: Box plots comparing GSN peptide integrated peaks, between never smoker workers occupationally exposed to SHS (NSE) and not exposed (NSNE).



Discussion

The present work is included in a series of studies, aiming to assess the impact of the tobacco legislation (law 37/2007), towards the protection of the workers occupationally exposed to SHS in Lisbon restaurants. The first study proved that the smoking designated areas in these venues were still highly polluted with tobacco related particles. It also demonstrated that workers, in this designated areas, were inhaling tobacco contaminants, given the significantly higher cotinine levels in their urine, compared with workers from non-smoking areas (Pacheco et al., 2012). The second study focused the workers health. A clinical evaluation revealed that all workers were healthy and no significant oxidative stress differences were found. Nonetheless at DNA level, the SHS exposed workers revealed a significant increased capacity to repair DNA lesions, after a challenge with a mutagenic agent (Louro et al., 2011). Also, nine plasma proteins were significantly changed. Most of these proteins are involved in acute phase inflammation response (Pacheco et al., 2013). In order to corroborate and complement the plasma proteomic results, instead of pooling the samples, we aimed to identify candidate biomarkers by applying an individual plasma profile analysis in a mass spectrometer featuring high resolution, mass accuracy, and dynamic range. With this technique, we were able to identify 521 proteins, where 11 were significantly differentiated in SHS exposed workers.

Functional annotation revealed that these proteins are involved in several different processes including regulation of immune system, apoptosis, aging, regulation of transcription, proteolysis, glycolytic process, phosphorylation, epithelial cilium movement, transport, cell adhesion, blood coagulation, acute-phase response, and binding to calcium and metal ions (Binns et al., 2009). Gelsolin (GSN), a protein already flagged in the previous plasma study, due to a significant decrease in SHS exposed workers (Pacheco et al., 2013), is involved in pathological processes associated with tobacco-related diseases, including chronic obstructive pulmonary disease (COPD) and lung cancer (Bortner et al., 2011). Protein alpha-1-acid glycoprotein 2 (ORM2) was also prominent given its statistical relevance and the fact that, together with CP and ITIH4, it belongs to acute phase inflammation reaction proteins. The former were already cited for their possible critical role in the SHS response mechanism (Pacheco et al., 2013).

Proteomic expression profiling studies have been increasingly used to discover and characterize single biomarkers for different purposes. However, without further validation, most are dismissed, due to the apparent lack of specificity (Hudler, Kocevar, & Komel, 2014). The solution might be in the quantitative analysis of a panel of proteins and SRM is a particularly suitable validation technique for these situations (Ivancic, Irving, Jonakin, Dove, & Sussman, 2014). Indeed SRM has been demonstrated to precisely quantify a set of proteins, in a consistent and reproducible manner, across multiple samples (Picotti & Aebersold, 2012 and Addona et al., 2009).

After performing SRM validation, two peptides from GSN and ORM2, were confirmed to be significantly increased in SHS exposed, EVQGFESATFLGYFK and WFYIASAFR, respectively. Overall ORM2 peptides were increased and GSN recorded two increased peptides and three decreased. Interestingly, all peptides from GSN further away from the N-terminus were decreased in SHS exposed workers, in contrast with the peptides closer to the N-terminus, that were increased. Opposite expression pattern of a protein in the same study condition was already observed in the previous pooled plasma experiment (Pacheco et al., 2013); where low-molecular-weight isoforms (shorter polypeptide chain) of CP and ITIH4 were increased in SHS exposed non-smoker and high-molecular-weight isoforms (larger polypeptide chain) were decreased.

Based in the SRM principle that peptides are protein surrogates, given that the peptides further away from the N-terminus were decreased in SHS exposed workers, one might assume that existing high-molecular-weight isoforms of GSN would be decreased in SHS exposed workers, compared with the unexposed; and low-molecular-weight isoforms would be increased. This pattern suggests that SHS might induce a specific proteolytic cleavage or an increased instability, possibly due to oxidative modifications (Pacheco et al., 2013). In fact, there was an increased level of the oxidative marker 8-Hydroxydeoxyguanosine (8-OHdG) in SHS exposed workers, even though it was not significant. Also, at a DNA level, there was no significant difference in the repairing capacity before challenging the DNA with a mutagenic agent.

The same principle applies for potential low-molecular-weight isoforms of ORM2, given that both peptides near the N-terminus were increased in SHS exposed workers. The fact that no peptides further away from the N-terminus were selected for SRM validation, refrain any assumption for potential high-molecular-weight isoforms of

ORM2. However, together with CP and ITIH4, ORM2 belongs to acute phase inflammation reaction proteins, which strongly supports that they might have a similar behaviour after SHS exposure.

Although ORM was one of the proteins selected for immunodepletion in the first stage of the workflow, given the consistency of the results in both the high throughput analysis and the SRM validation assay, it was decided to keep ORM2 in the study.

Acute-phase inflammation proteins increase their plasma concentrations in response to inflammation. This group of proteins were already referred on literature as exhibiting abundance variation in SHS related diseases, but the exact pattern induced by SHS remains unclear.

So far, only a few data is available concerning the specific isoform ORM2. However a significant increase of ORM2 plasma levels in stage II colorectal cancer patients was described (Gao, Zhang, Whang, & Zheng, 2014). Another study suggest that ORM2 is a early potential biomarker for cholangiocarcinoma diagnosis (Rucksaken et al., 2012). In general, ORM proteins (either ORM1 or ORM2) were found to rise from lower levels in patients with early stages of breast cancer, to high levels in advanced cancer stage (Tesseromatis, Alevizou, Tigka, & Kotsiou, 2011). Furthermore, ORM plasma levels yielded a sensitivity of 89% and specificity of 84% in the detection of active lung cancer. During antineoplastic therapy, the normalization of ORM levels, correlated with a significantly prolonged relapse-free survival in lung and glioblastoma multiform cancer patients (Ganz, Baras, Ma, & Elashoff, 1984). Indeed, ORM was identified as potentially useful circulating biomarker, for estimating the five-year risk of cardiovascular, nonvascular, and cancer mortality (Fischer et al., 2014). Accordingly, its levels were significantly higher after myocardial infarction (Freilich & Giardina, 1984) and in smoking individuals (Angelika E. Stillbauer, 1983). As for involuntary exposure to SHS, Masauki and colleagues found a significant rise in ORM levels in children that are exposed at home compared with unexposed (Shima & Adachi, 1996).

As for GSN, human transitional cell carcinomas of the bladder frequently reveal chromosomal abnormalities where GSN encoding gene is localized, even at early stages. Tanaka and colleagues, found undetectable or extremely low GSN expression in tumor, compared with normal bladder epithelial cells and tissue. Furthermore, the introduction of exogenous GSN cDNA into a human bladder cancer cell line, greatly

reduced the tumorigenicity *in vivo* (Tanaka et al., 1995). Since most bladder and lung cancers have been associated with smoking, the same group examined GSN expression in a number of lung cancer cell lines. All revealed low-to-undetectable expression of GSN at both gene and protein level, compared to that in normal lung tissue. Moreover altered GSN expression significantly correlated with heavy smoking habit (Dosaka-Akita et al., 1998).

Proteomic studies on bronchoalveolar lavage fluid (BALF) revealed much less GSN in Pulmonary Langerhans cell histiocytosis (PLCH) patients, compared with both healthy and COPD (Ghafouri, Persson, & Tagesson, 2013). Another study found up-regulated GSN in the plasma of healthy smokers compared to healthy non-smokers (Bortner et al., 2011).

Together with our results, these studies suggest a role of ORM and GSN in pathological mechanisms related with tobacco smoke exposure, which makes them serious targets for future studies

Conclusion

Tobacco smoke is by far one of the leading causes of lung cancer and death.

Despite remarkable progress in proteomic methods, currently there is no biomarker sufficiently powered to use in early detection of lung cancer or other diseases, related with chronic exposure to SHS.

In this proteomic work, by accurate, complementary and targeted mass spectrometry approaches, we reinforce the importance of host response proteins previously identified, and present new candidate biomarkers of SHS exposure. A detailed study of the common expression pattern and the molecular mechanisms where this panel of CP, ITIH4, GSN and ORM is evolved, might lead the way towards SHS disease prevention, or improve earlier diagnosis.

Additionally, these findings encourage decision makers to urgently ban completely tobacco smoke form all indoor spaces to effectively protect workers from SHS exposure.

4th study

Secondhand smoke - From indoor air to epithelial cells

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Abstract

Tobacco-related sickness and death place it on the top of global burdens. Secondhand smoke (SHS) accounts for part of this burden and the workplace is one of the main sources of SHS exposure. A previous study revealed high levels of tobacco smoke contaminants in smoking designated areas in Portuguese restaurants. Additionally these venues workers recorded significant changes at the DNA and protein level, after the confirmation that they were inhaling SHS. Most of these proteins are involved in tobacco smoke-induced cardiovascular and respiratory disease.

The lack of biomarkers for early diagnosis of SHS related illness, driven the present work which aims to find candidate biomarkers of SHS exposure in nasal epithelium of healthy occupationally exposed workers, that never smoked.

Following a proteomic approach, 21 proteins were significantly different. Most of these proteins were already related with common outcomes of tobacco smoke, such as COPD and lung cancer.

Together with the chronic nature of occupational exposure, these ongoing subclinical molecular mechanisms might be missed and end up in preventable disease,

or even death. Therefore these candidate biomarkers should be further validated for future application in routine occupational health screening.

Introduction

Tobacco is expected to cause as many as one billion deaths worldwide in this century (WHO, 2013).

Tobacco smoke contains thousands of chemicals, including over 70 known to cause cancer (NCI, 2011). Smoking is the major cause of the most deadly type of cancer, the lung cancer. In Europe, lung cancer accounts for approximately 20% of all cancer deaths. In the US, every year, smoking causes about 90% male- and 80% of female- lung cancer deaths (Stephen S. Hecht, 1999). Numerous studies associate lung cancer risk with chronic obstructive pulmonary disease (COPD), and exposure to tobacco smoke is a common source for both pathologies (WHO, 2012a).

Evidence also infer that both active and involuntary smoking, cause coronary heart disease. Indeed, even at low levels of tobacco smoke exposure, cardio-vascular disease risk rise rapidly (Teo et al., 2006).

Although progress has been made to increase protection from SHS, almost 50% of non-smokers, and more than 60% of young children, continue to be exposed to SHS (NIOSH, 2015). These numbers correspond to near 1.8 billion non-smokers at risk.

Exceptions to smoke-free legislation, for example in public venues where guests are allowed to smoke in designated areas, constitute major health risk. A previous study in Portuguese venues, revealed that these areas have a significant high level of SHS contaminants, regardless of ventilation and other protective measures. Furthermore, workers in these areas were inhaling polluted air, and revealed consistent changes in the DNA and in a panel of plasma proteins. Therefore it is urgent to better identify these subjects at greatest risk. Moreover, it is crucial to develop effective methods for early detection of these molecular changes, while tobacco smoke-related diseases are not established yet (Dubey & Powell, 2008).

The use of molecular profiling, and assessments of proteins, are among the preferential choices to develop biomarkers at early stages of disease. In addition, it allow a better understanding of the molecular and cellular pathways involved in the pathological process (Alberg, Brock, & Samet, 2005).

Previously a plasma profiling study identified 521 proteins, including 11 that were significantly different in SHS exposed workers. These proteins were involved in mechanisms underlying several tobacco smoke-induced diseases, such as acute phase inflammation response, oxidative stress and protease-antiprotease imbalance. In order to better understand these molecular mechanisms induced by SHS exposure, and search for protein biomarkers, we performed a protein profiling study at the respiratory level. Given the inherent difficulties to have access to bronchial epithelia, we collected nasal epithelial cells, that were already demonstrated to be a suitable model to study lung diseases (Simões et al., 2011). Besides being easily collected, this bio specimen is in constant contact with tobacco smoke pollutants.

Despite all the scientific evidence on the tobacco smoke adverse health effects, many gaps still remain. With this study we expect to find candidate biomarkers in nasal epithelia and hence contribute for more cost-effective routine screening and preventive therapies, that block, or reverse, the underlying process of injury. Additionally we expect to contribute with more insights on the early molecular mechanisms of tobacco smoke-induced diseases.

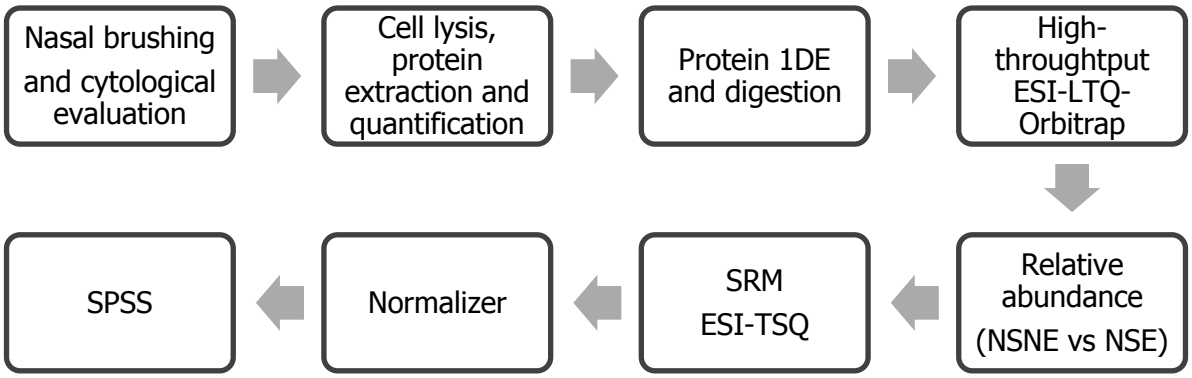
Material and Methods

Sodium dodecyl sulfate (SDS), Glycine, Tris(hydroxymethyl) aminomethane (Tris Base) from GE Healthcare (Uppsala, Sweden). Phosphate buffer saline (PBS), Formaldehyde, May-Grünwald solution, Dithiotreitol (DTT) and Trifluoroacetic acid (TFA) from Sigma Aldrich and GelCode Blue Stain purchased from Nordic Biolab (Stockholm, Sweden). Laemmli sample buffer, 12% Criterion TGX Gel, 12+2 well, 45 µl and Precision Plus Protein Standard from Bio-Rad (Hercules, CA). Sequencing grade modified trypsin from Promega Biotech AB (Nacka, Sweden). Ethylenediaminetetraacetic acid (EDTA), Acetonitrile (ACN) and Ammonium bicarbonate (AmBic) from Fluka (Sigma Aldrich, Stockholm, Sweden). BCA Protein assay kit from Pierce Chemical Company (Nordic Biolab, Stochholm Sweden). Formic acid (FA) from J.T. Backer VWR (Sigma Aldrich Stockholm, Sweden). Iodoacetamide (IAM) from AppliChem and sodium chloride (NaCl) from Honeywell (Saveen Werner, Malmö Sweden). Tris (hydroxymethyl)aminomethane hydrochloride (Tris-HCL) from VWR (Merk) OneMed Group (Lund, Sweden). Acetonitrile (ACN) and water for HPLC from

Merck (Solna, Sweden). Bristles from Paro-Isola, (Thalwil, Switzerland). SpikeTides Peptide Technologies GmBH acquired from JPT Peptide Technology (Berlin, Germany).

The study workflow is depicted in Figure 15.

Figure 15: Workflow.



From nasal epithelial cells collection to cytological evaluation

Following informed consent, cells from the nasal epithelia (NE) were collected from 27 individuals, through a brushing procedure previously described (Ramalho et al., 2003). Briefly, an interdental brush with up to 3 mm bristles was used to scrape along the tip of the inferior turbinate and the adjacent lateral nasal wall. Brushes containing cells were immediately placed into an end-cut automatic pipette tip inside a plastic tube with PBS pH 7.4 at room temperature (RT). Cells were removed from the brush by flicking it up-and-down inside the tip and collected by centrifugation at 3,000 rpm for 4 min. Most of the supernatant was wasted and, before storage, an aliquot was removed for cytological examination. The aliquoted cells were re-suspended in fixing solution containing 3.7 % mass/volume (m/v) sucrose and 4% formaldehyde at 4ºC. Then, following the procedure previously described (Penque et al., 2000), cells were spread on silane glass slides and stained by the May-Grünwald-Giemsa method (Bain & Mitchell Lewis, 2006). The samples on slides were evaluated on a conventional light microscope (Zeiss, Jena, Germany) at 100 X magnification under oil immersion. Cells were identified on the basis of morphology and classified as epithelial cells according with criteria described by other authors (C Danel, 1996). All cells that did not meet the

criteria were excluded from this group. One subject was excluded from the study due to sample contamination.

From proteins to peptide extraction

Cells from each sample were disrupted by a sequence of vortexing (during 1 min at 4°C in 130 µl of lysis buffer containing 10 mM Tris-HCl pH 7.4, 2 mM EDTA, 1% SDS and 20 mM NaCl), 2 min heating at 95°C plus 3 min sonication bath at RT. To ensure maximal cell lysis, samples were left in lysis buffer overnight at 4 °C and then spun down at 16,1 g (for 1 min at 4°C) to sediment membranes and other debris. The supernatant was collected to a new tube, centrifuged at 16,1 g (for 1 min at 4°C) and protein quantity was estimated using the BCA protein assay kit. After speed-vacuum dehydration, 26 samples with 10 µg of protein, were separated by SDS-PAGE. Twenty microlitre of laemmli sample buffer (containing 62.5 mM Tris-HCl at pH 6.8, 25% glycerol, 2% SDS and 0.01% Bromophenol Blue) and 350 mM DTT were added to each sample. After vortex and quick centrifugation at 16,1 g (for 20 sec at 4°C), samples were heated at 95°C during 5 min. Then proteins migrated until they were into one single lane, in 12% precast gels (applying 100 V constant current, for 20 min at RT). Later the gels were washed 3 times, stained using the GelCode blue stain reagent and each lane was cut manually into nine squares of $1mm^2$. The slices were distained (in 50% ACN and 25 mM AmBic, during 30 min at RT, twice), before performing protein reduction (using 10 mM DTT in 100 mM AmBic) during 1 h at 57°C. Protein alkylation was performed by incubating slices in 100 mM AmBic and 55 mM IAA (for 45 min at RT, in the dark). Slices were then washed twice with 100 µL of, in sequence, 100 mM AmBic followed by ACN 100%; speed-vac dehydration and incubated (for 12 h at 37°C) with 150 µl trypsin (12,5 ng/µl in 50 mM AmBic).

Peptides were extracted from the gel slices by incubation in 200 µL of 75% ACN and 5% TFA during 30 min at RT, under vortexing. To increase the yield of peptide extraction, the latter step was repeated twice, followed by the incubation with 200 µL of ACN 100% for 10 min and speed vac dehydration. Samples were stored -80ºC until further analysis.

Liquid Chromatography coupled with Mass Spectrometry

To allow an efficient LC-MS/MS analysis, the 26 samples were resuspended in 0,1% FA (for 30 min) and subjected to C18 spin column-based cleaning procedure. An ESI-LTQ-Orbitrap XL mass spectrometer (Thermo Fisher, Bremen, Germany) interfaced with an Eksigent nanoLC 2DTM plus HPLC system (Eksigent technologies, Dublin, CA, USA) was used for all samples. The auto-sampler injected 5 µL (a total of 2.5 µg of protein digests). A blank LC-MS/MS run was performed between each analyzed sample. Peptides were loaded, trapped and washed with a constant flow rate of 10 µl/min with solvent A (composed with 0.1 % FA), for 15 min onto a pre-column (PepMap 100, C18, 5 µm, 5 mm x 0.3 mm, LC Packings, Amsterdam, Netherlands). The peptides were subsequently separated on a 10 µm fused silica emitter, 75 µm x 16 cm (PicoTipTM Emitter, New Objective, Inc.Woburn, MA, USA), packed in-house with Reprosil-Pur C18-AQ resin (3 µm Dr. Maisch, GmbH, Germany). Peptides were eluted with a flow rate of 300 nl/min with a 60 min linear gradient of 3 to 35% (v/v) ACN in water, containing 0.1% (v/v) FA and then increased rapidly to 90% (until min 68) and was kept until min 78, before re-equilibrating. The LTQ-Orbitrap was operated in DDM to automatically switch between Orbitrap-MS (up to 2,000 Da) and LTQ-MS/MS (between 50 to 2,000 Da) acquisition. Mass spectra of all samples were always acquired in the positive ionization mode. Four MS/MS spectra were acquired in the linear ion trap per each FT-MS scan, which was acquired at 60,000 FWHM nominal resolution settings using the lock mass option (m/z 445.120025) for internal calibration. The dynamic exclusion list was restricted to 500 entries using a repeat count of two with a repeat duration of 20 sec and with a dynamic exclusion range of 2 min after the second peptide count. Precursor ion charge state screening was enabled to select for ions with at least two charges and rejecting ions with undetermined charge state. The CID energy was set to 35%, and one micro scan was acquired for each spectrum. The complete study was run using four days of MS-instrumentation time. A total of 26 LC-MS/MS runs were performed.

Mass spectral data analysis

All LC-MS/MS data were analyzed in Progenesis, version 2.10.0, which performs automated quantification of peptide abundance. MS/MS spectra were searched, against

the UniProt human database (including a decoy data set) using Mascot search (Matrix Science). In the search parameters, the enzyme was set to trypsin, with two miss cleavages allowed and peptide and fragment mass tolerances of 10 ppm and 0.6 Da, respectively. Carbamidomethylation of cysteine and the oxidation of methionine were set as fixed and variable modifications respectively and a 0.01 false discovery rate (FDR) threshold was selected at both protein and peptide levels. Protein ratios were calculated from the combined value of non-conflicting peptide values and combined for similar proteins. A t-test was used to evaluate the abundance difference obtained between the study group samples, with a p-value of <0.05 being considered as statistically significant. Significant proteins were further selected for SRM validation based on their fold change.

Specific Reaction Monitoring

In order to quantify a target protein, an SRM assay was developed in Skyline, version 2.6 (MacLean et al., 2010). The peptides were checked against a background, with the entire human proteome (UniProt KB download) and included only those with 7-25 amino acid length. Synthetic peptides, were used, to validate the peak selection. The peptides were solubilised according to manufacturer's instructions and a pool of peptides, with a final concentration of 250 fmol/µL, was constructed. Two microlitre of this pool were spiked in 8 µL NE peptide samples. An Eksigent 2D NanoLC system (Eksigent technologies, Dublin, CA, USA) connected to a Triple Stage Quadrupole Mass Spectrometer (TSQ Vantage, San José, CA), with a nano electrospray interface, was used for LC-SRM analysis. Solvents used were mobile phase A (mobA) and B, which is, water/0.1% FA (v/v) and ACN/0.1% FA (v/v), respectively. The LC system was equipped with a trap column (0.3 mm id, 5 mm, PepMap Acclaim C18, LCPackings, Sunnyvale, CA) and in-house packed analytical columns, in 0.075 mm id, 100 mm PicoFrits (New Objective, Woburn, MA), with 3 µm ReproSil C18-AQ particles (Dr. Maisch, Germany), to a length of 12 cm. A 300 nl/min linear LC gradient delivered mobA, starting with 97%, then decreasing to 85% during 6 min, and then further down to 65% during 68 min. An injection, for wash and equilibration of the column, was performed between each sample. The TSQ operated in the positive ion mode, with a

spray voltage of 1.8 kV and an ion capillary temperature of 270°C. A 0.7 Da unit resolution, for both Q1 and Q3 were set as well as 10 ms dwell time per transition.

Data were acquired using the Xcalibur version 2.0.7 and the output raw files were re-imported to Skyline, to be analyzed and manually inspected. Peak areas, for the measured SRM transitions for each individual peptide, were integrated and summed to generate the peptide peak areas. Data was exported from Skyline as .csv files. After data normalization (Chawade et al., 2014) it was imported to IBM SPSS, version 22, for statistical testing. Normally distributed data was analyzed with two-sided Student's t test.

Results

In order to study the molecular mechanisms induced by SHS exposure, before the first symptoms of related diseases arise, a group of healthy never-smoker workers was recruited. Overall this study included 27 subjects from 25 Lisbon restaurants. They were separated in two groups, according with their occupational exposure to SHS. Therefore, 14 were working in smoking designated areas and other 13 were working in smoke-free areas. After a detailed lifestyle survey and a clinical evaluation (Pacheco et al., 2013) the NE samples were collected from 26 never-smoker workers and mostly (77%) were males (Table 14). Half were exposed to SHS in the workplace, nearly 38h in a week, for the last eight years. The remaining, work approximately 46 h in a week, in currently smoke-free environments.

Table14: Mean age, body mass index (BMI) and time that never-smoker subjects spend at the workplace, according with their occupational exposure to SHS.

	Not-exposed to SHS	**Exposed to SHS**
Age	43	**34**
BMI	27.0	**25.7**
Males	8	**12**
Working Hours in a week	46	**38**
Working years	7	**8**
	N=13	**N=13**

Shotgun analysis identified a total of 17,561 peptides corresponding to 3,384 proteins.

Table 15: List of the nasal epithelial proteins that significantly differentiated SHS exposed workers.

Accession nº	Description	Gene name	p value	Fold change
B7Z242	Amine oxidase [flavin-containing] B	MAOB	0.04	-1.8
Q86UP2-1	Isoform 1 of Kinectin	KTN1	0.05	-1.7
Q14210	Lymphocyte antigen 6D	LY6D	0.05	-2.3
P42768	Wiskott-Aldrich syndrome protein	WAS	0.05	-1.6
P26038	Moesin	MSN	0.02	-1.3
P26373	RPL13 protein	RPL13	0.03	-2.8
B4DPP0	cDNA FLJ51032, highly similar to CD9 antigen	na	0.03	-2.4
B2RBR9	cDNA, FLJ95650, highly similar karyopherin (importin) beta 1 (KPNB1), mRNA	na	0.05	-1.8
B3KQB4	cDNA FLJ90131 fis, clone HEMBB1000447, highly similar to PRA1 family protein 3	na	0	-1.3
B3KVX6	cDNA FLJ41699 fis, clone HCHON2004776, highly similar to cytoskeleton-associated protein 4 (CKAP4), mRNA	na	0.02	-1.4
O95782-1	Isoform A of AP-2 complex subunit alpha-1	AP2A1	0	1.4
B0YIW5	Archain 1, isoform CRA_b	ARCN1	0.01	1.3
P53621-1	Isoform 1 of Coatomer subunit alpha	COPA	0	1.5
E9PEL9	Nesprin-1	SYNE1	0.02	1.3
P13639	Elongation factor 2	EEF2	0.02	1.3
H0YAF8	Guanine nucleotide-binding protein subunit beta-2-like 1 (Fragment)	GNB2L1	0	1.3
H9ZYJ2	Thioredoxin	TXN	0.04	1.7
Q99714-1	Isoform 1 of 3-hydroxyacyl-CoA dehydrogenase type-2	HSD17B10	0.03	1.3
O60763-1	Isoform 1 of General vesicular transport factor p115	USO1	0.02	1.2
P62241	40S ribosomal protein S8	RPS8	0.04	1.3
Q1AHP8	Hepatopoietin PCn127	na	0	1.3
Q5T6L4	Argininosuccinate synthetase, isoform CRA_a	ASS	0.04	1.4
M0QXB5	Persulfide dioxygenase ETHE1, mitochondrial	ETHE1	0.05	1.4
Q9BTT5	Similar to NADH dehydrogenase (Ubiquinone) 1 alpha subcomplex, 9 (39kD) (Fragment)	na	0.02	1.3
B4E1R1	cDNA FLJ52389, highly similar to Alcohol dehydrogenase 1B (EC 1.1.1.1)	na	0.04	1.6
B4DMY3	cDNA FLJ60713, highly similar to heterogeneous nuclear ribonucleoprotein A/B (HNRPAB), transcript variant 1, mRNA	na	0.03	1.3
B4DH02	cDNA FLJ50510, highly similar to Heat shock 70 kDa protein 4	na	0.02	1.4

After statistical comparison, 27 proteins were significantly different in SHS exposed workers (Table 15). Among those, ten were less abundant in the SHS exposed group (negative fold change in Table 15).

In order to validate the results an SRM analysis was performed. Time and financial constrains restricted the validation. Two over-expressed and two under-expressed proteins were firstly selected, namely Lymphocyte antigen 6D (LY6D), 60S ribosomal protein L13 (RPL13), Elongation factor 2 (EF2) and 40S ribosomal protein S8 (RPS8).

After manual selection of the best transitions, a final SRM assay was constructed with four proteins, nine corresponding peptides and 45 transitions (Supplementary information: Table 21). Samples were spiked with the synthetic version of the naturally occurring peptides. After total intensity normalization, peptide integrated peak areas were compared between SHS-exposed and unexposed workers.

Four peptides, belonging to RS8, EF2 and RPL13, were significant. All peptides, from the same protein, behaved in the exact same way within each study group and all SRM results match previous MS shotgun analysis results (Table 15). Therefore, in SHS exposed workers all peptides from RS8 and EF2 were increased and all peptides from RL13 and LY6D were decreased, compared with unexposed workers (from Figure 16 to 19).

Figure 16: Box plots comparing RS8 peptide integrated peaks, between never-smoker workers occupationally exposed to SHS (NSE) and not-exposed (NSNE).

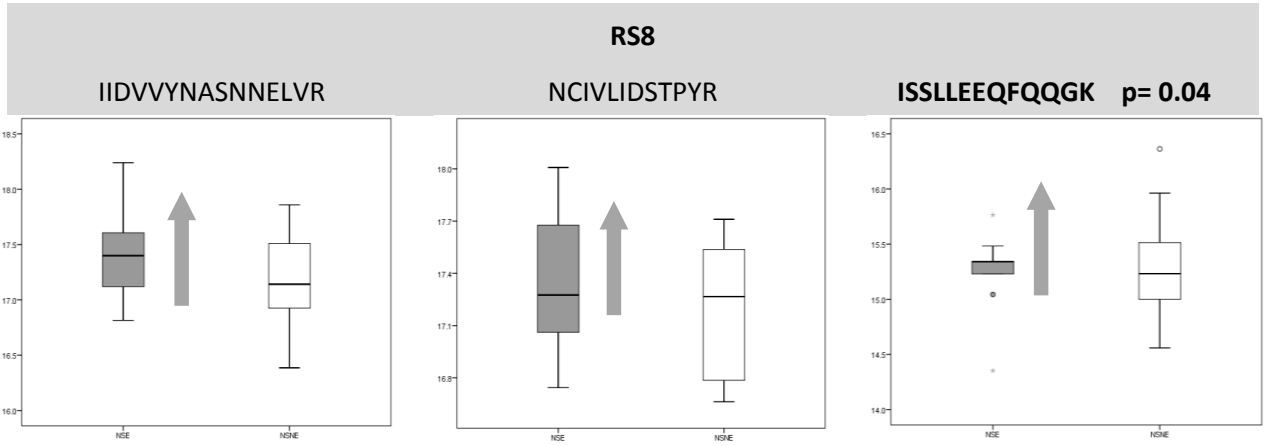


Figure 17: Box plots comparing EF2 peptide integrated peaks, between never-smoker workers occupationally exposed to SHS (NSE) and not-exposed (NSNE).

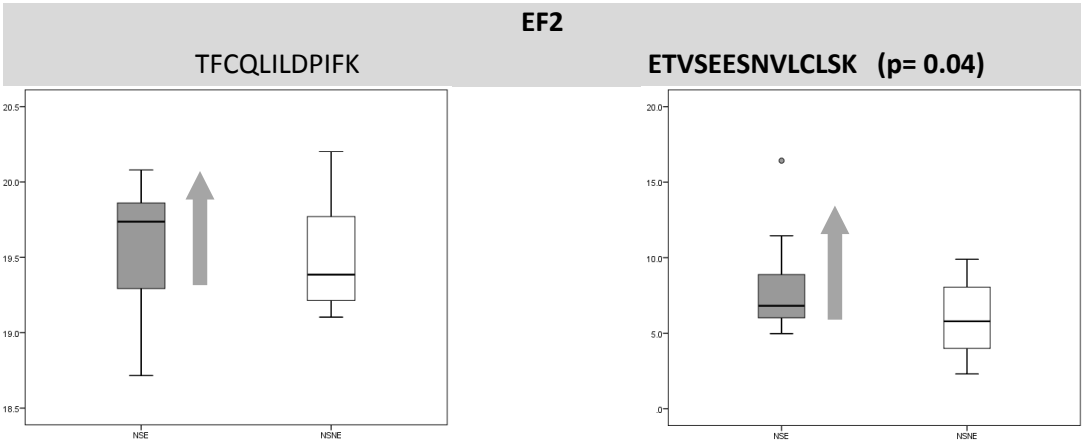


Figure 18: Box plots comparing RL13 peptide integrated peaks, between never-smoker workers occupationally exposed to SHS (NSE) and not-exposed (NSNE).

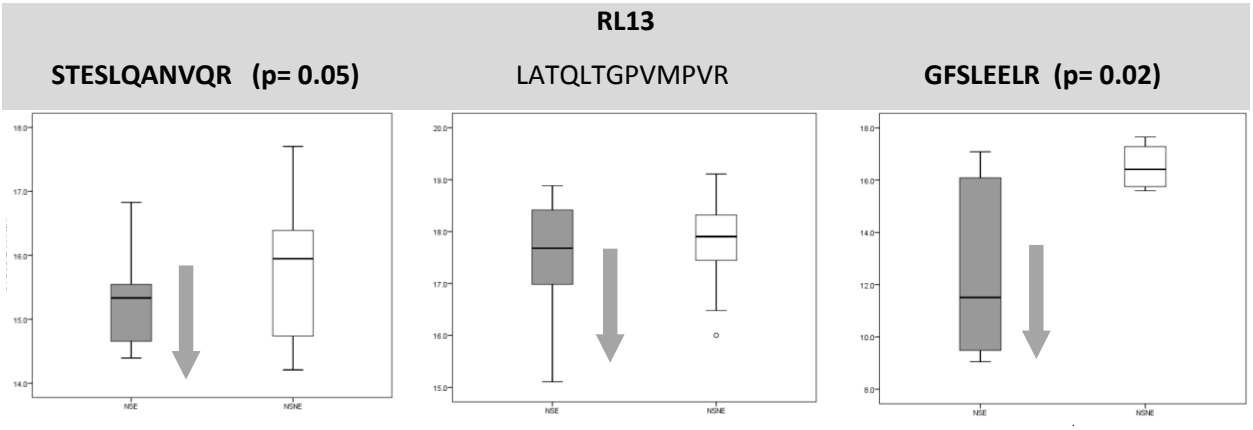
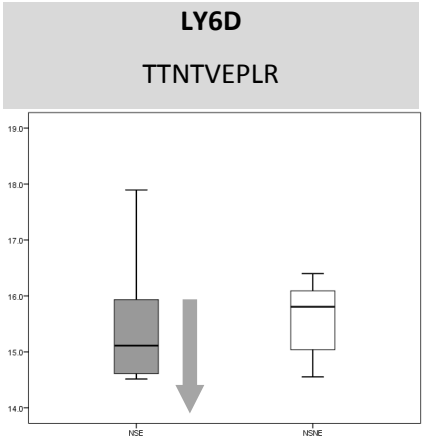


Figure 19: Box plots comparing LY6D peptide integrated peaks, between never-smoker workers occupationally exposed to SHS (NSE) and not-exposed (NSE).



Discussion

SHS is the dominant form of indoor air pollution in spaces where tobacco is smoked, even if properly ventilated (WHO, 2010). Due to the health risks posed by SHS, health authorities worldwide advise to reduce exposure to the lowest levels. The vast time spent at the workplace makes it one of the main sources of SHS exposure.

In Portugal, the ambition to protect both personnel and clients from SHS, prompt the tobacco Law 37/2007. Accordingly, a number of changes occurred in public venues and some became entirely smoke-free or mixed, including smoking and non-smoking designated areas inside their premises.

The will to understand if the new law is effectively protecting Portuguese venues workers from SHS exposure, motivated this work.

Preliminary results revealed that smoking-areas were highly polluted with SHS and contaminating adjacent non-smoking areas. Furthermore, the workers from smoking areas were inhaling SHS, had a higher DNA repairing capacity and a significant difference in a group of plasma proteins, including CP, ITIH4 and GSN (Louro et al., 2011; Pacheco et al., 2012, 2013). Among others, these plasma proteins are involved in a series of molecular processes related with tobacco smoke-induced diseases. The present work aims to follow these subclinical molecular processes.

Plasma and serum are easily collected and interact with the majority of body tissues. As a result, they are considered keystones in numerous medical methods. On the other hand, there is increasing evidence of the critical role played by the bronchial epithelium in airway homeostasis, due to its unique situation at the interface with the environment (Gras, Chanez, Vachier, Petit, & Bourdin, 2013). Thus, bronchial and lung biopsies, together with induced sputum, bronchoalveolar lavage, nasal lavage fluid and exhaled breath condensate, have been the centre of respiratory disease-studies. Still, the difficulty of these procedures motivated the use of more accessible alternative samples. In fact, proteomic studies revealed a considerable number of similarities between bronchial and nasal epithelium (NE) which makes the last a reliable surrogate to measure xenobiotic responses in bronchial tissue (Penque et al., 2000; Simões et al., 2011; Simões, 2012; Iskandar et al., 2013 and Jeanson et al., 2014). In addition, NE is easily collected by well tolerated brushing technique, avoiding aforementioned invasive procedures.

Accordingly, in order to strengthen the evidence that workers in smoking designated areas are indeed undergoing SHS-induced molecular mechanisms, the present work focus their respiratory system, specifically the NE proteome.

This study included 26 never-smoker workers, half were working in smoking designated areas and hence, were exposed to SHS (Table 14).

The proteome profiling MS analysis identified 3,384 proteins. Among those, 27 were significantly different in SHS exposed workers, specifically ten were decreased while 17 were increased (Table 15).

Besides cytological evaluation, the epithelial origin of most cells was confirmed by the proteins identified including mucin 1, 4, 6, 7, 5AC and 5B, keratin type I, II, cytoskeletal 8, 14 and epithelial cell adhesion molecule. All are reported to be expressed at relatively high levels in the human respiratory tract (Banks-Schlegel, McDowell, Wilson, Trump, & Harris, 1984; Cole et al., 2010; Reid, Gould, & Harris, 1997; Aust, Madsen, Jennings, Kasperbauer, & Gendler, 1997; Thornton et al., 2000; Rogers, 2000; Yoon & Park, 1998 and Yuan, Shyy, & Martins-Green, 2009).

After additional MS validation procedure, proteins EF2, RS8 and RPL13 were confirmed to be significantly changed in SHS exposed workers compared with the unexposed (from Figure 16 to 19). Furthermore, the perfect match between the validation and the profiling results indicate that LY6D (Figure 19), together with other significant, but not yet validated proteins, might also be NE candidate biomarkers of SHS exposure.

A detailed study of these proteins revealed substantial information to better comprehend the molecular response to SHS exposure.

Tobacco smoke is made up of more than seven thousand chemicals (NIOSH, 2015). Polyaromatic hydrocarbons (PAH) and acrolein are included and cause irreversible changes that might either kill the cell or cause a genetic mutation (Shihadeh et al., 2015). Previous studies, in these public venues predicted significantly higher levels of particulate bounded-PAH in smoking designated areas (Pacheco et al., 2013). Compared to PAH, acrolein might be a carcinogen in a higher extent, particularly in the lungs (Chen, Fang, Li, Tang, & Jin, 2013). In fact it is more abundant in tobacco smoke and is also a endogenous product of oxidative stress (Chen et al., 2013). Additionally, it is highly reactive towards lysine, arginine, histidine, and cysteine residues within

susceptible proteins (Aldini, Orioli, & Carini, 2011). Accordingly, workers from smoking-designated areas recorded a significant increase in a mitochondrial protein involved in lysine acetylation, persulfide dioxygenase (ETHE1). Besides cancer, lysine acetylation is also implicated in Multiple sclerosis, Alzheimer and pulmonary and cardiovascular diseases (Chen et al., 2013). Understanding these epigenetic mechanisms, whereby environmental factors, such as SHS exposure, interact with the genome and proteome, may prevent irreversible alterations in numerous cellular processes.

Other two mitochondrial proteins, similar to NADH dehydrogenase (Ubiquinone) 1 alpha subcomplex, 9 (39kD) (Fragment) and Isoform 1 of 3-hydroxyacyl-CoA dehydrogenase type-2, were significantly increased in SHS exposed workers compared with the unexposed. Mitochondria is responsible for generation of most usable energy in the cell. It also plays an important role in a series of physiological processes, including apoptosis. Ubiquinones are hydrogen carriers and function as ubiquitous coenzymes in redox-reactions (Consortium, 2015). A global proteomic analyses of nasal epithelial cells, comparing smokers with non-smokers, also recorded significant higher levels of this protein (Alexandre, 2011). Also, a study revealed that mitochondrial NADH dehydrogenase was significantly modulated by nicotine in multiple brain regions (Wang, Kim, Donovan, Becker, & Li, 2009).

Another prominent protein with a significant increase due to SHS exposure, was argininosuccinate synthetase, isoform CRA. An *in vitro* study revealed that this protein generally mirrors the induction of nitric oxide (NO) synthase. Also a study in asthma patients found increased arginine levels in airway epithelial cells, which supports the high rate of NO synthesis in these patients (Marczin & Yacoub, 2002). Conversely in cigarette smokers NO levels are chronically decreased (Schilling et al., 1994). NO is a gaseous mediator which has an important role in several physiological processes in the respiratory tract. It may be either a protective or a contributing factor to oxidant-induced cytotoxicity to respiratory tract. In fact, smoking is related with increased oxidative stress and NO production, both contributing to lung injury (Balint, Donnelly, Hanazawa, Kharitonov, & Barnes, 2001). Nonetheless, in active-smokers, exhaled NO might be reduced, as pulmonary antioxidant defence mechanisms take effect, because it is trapped at the airway epithelial surface (Chambers, Tunnicliffe, & Ayres, 1998).

Nesprin-1 was also increased in SHS-exposed workers. This protein family maintain nuclear organization and structural integrity of the cell (Zhang et al., 2001). Nesprin-1 gene, SYNE1, is the first identified gene responsible for a recessively inherited pure cerebellar ataxia (Gros-Louis et al., 2007). Kita and co-workers reported that nicotine also induce ataxia (Kita, Nakashima, Shirase, Asahina, & Kurogochi, 1988). Interestingly, following SHS exposure, there was a significant up-regulation of SYNE1 in the hippocampus of mouse embryos. The same study postulated that SHS exposure *in utero* may enhance hippocampus expression of SYNE1. Together with the ataxia-inducing effect of nicotine, it may turn the embryos susceptible to ataxia and associated neuropathies (Mukhopadhyay, Horn, Greene, & Pisano, 2010).

Among the up-regulated proteins listed, there was a highly similar to heat shock 70 kDa protein 4. Environmental stress factors, such as tobacco smoke exposure, induce heat shock proteins in the airways. This stress mechanism is supposed to activate the innate immune system, resulting in airway inflammation (Doz et al., 2008 and Dong et al., 2013). Accordingly, a number of inflammation proteins were already flagged due to their significant increase in our previous plasma results, namely CP, ITIH4 and ORM2. Also, in a recent study, the levels of heat shock proteins were inversely correlated with a serum marker of oxidative stress, 8-Oxo-2'-deoxyguanosine (X. Wu et al., 2013). Our study found no significant changes of this stress biomarkers, although there was a slight increase in SHS exposed workers (Pacheco et al., 2013). Additionally, researchers found that long-term exposure to cigarette smoking might reduce expression of Hsp70 or increase auto-antibodies production, which may contribute to the development of airway diseases (Xie et al., 2010). Therefore, these auto-antibodies are considered as serological markers of smoke-related lung-diseases (Newkirk et al., 2012).

Analogous to Ceruloplasmin, other oxidoreductase proteins highly similar to amine oxidase and to alcohol dehydrogenase were significantly differentiated in SHS exposed workers (Consortium, 2015). Amine oxidase decreased abundance, might be related with the presence of beta-carboline alkaloids in cigarette smoke. The later act as potent reversible inhibitors of amine oxidase enzymes, reducing their activity in subjects exposed to tobacco smoke (Herraiz & Chaparro, 2005). Conversely alcohol dehydrogenase was increased in SHS-exposed workers. This enzyme catalyzes the

oxidation of ethanol into acetaldehyde. Increased dehydrogenase activity in SHS-exposed workers favours acetaldehyde production. Acetaldehyde is not appanage of tobacco smoke, it is ubiquitous in the environment. Hence, many individuals are exposed just by breathing ambient air. Additionally, drinking and smoking have a synergistic effect in alcohol metabolism (Yokoyama, Omori, & Yokoyama, 2010). Therefore, when associated with alcohol consumption, acetaldehyde is considered a carcinogen (IARC, 2015). A study in East Asian drinkers concluded that the combination of drinking and tobacco smoke exposure, together with less active alcohol dehydrogenase proteins, increase the risk of squamous cell carcinoma in the upper aero digestive tract (Asakage et al., 2007).

Thioredoxin also belongs to oxidoreductase family and is a primary defence in bronchial and alveolar epithelium. In this study this protein expression was elevated in SHS-exposed workers which may indicate ongoing cell regeneration and inflammation. In fact, increased antioxidant property due to thioredoxin over expression was documented to attenuate tobacco smoke-mediated oxidative stress and emphysema (Tiitto et al., 2003; Sato et al., 2008 and Rahman & Kinnula, 2012). During oxidative stress, thioredoxin binds to proteins, such as hepatopoietin, also increased in our study. Ultimately, this hepatopoietin - thioredoxin interaction activates the redox-sensitive transcription factor, suggesting a new redox-signal pathway in cytoplasm (Li et al., 2005). Conversely in high-grade tumours, thioredoxin expression was diminished, suggesting a loss of redox-regulation (Soini et al., 2001).

Kinectin isoform 1 was also down-regulated in SHS-exposed workers. Kinectins are selectively involved in the transport of specific types of organelles. Nevertheless, different cellular processes use specific kinectin isoforms (Santama, Er, Ong, & Yu, 2004). Also, the absence of a specific interaction with an elongation factor, favours synthesis of cytoplasmic proteins instead of membrane proteins (Ong, Lin, Zhang, Chia, & Yu, 2006). Therefore the distribution of elongation factors might be used to regulate protein synthesis (Sasikumar, Perez, & Kinzy, 2012). A thorough understanding of these alternate functions of elongation factors is essential. Indeed, elongation factor 2 was also relevant in our study, with a validated significant increase in SHS exposed workers. Translation elongation factors, are among the most abundant proteins in the cell, and play an essential role in assuring an appropriate cellular response to external stimuli.

Three RNA binding proteins were also highlighted in this study. Two, are ribosomal constituents and were inversely expressed in SHS exposed workers. Up regulated 40S ribosomal protein S11, is a component of the 40S ribosome subunit, whereas down regulated RPL14 is a constituent of the 60S subunit. Ribosome's, are organelles that catalyze protein synthesis (Consortium, 2015). In order to identify signalling pathways activated by tobacco smoke exposure in the acute phase, a recent study monitored a similar protein in the lung tissue of mice. The results revealed that it was associated with the response to stress and nicotine, as well as involved in a number of pathways including inflammation, repair, regeneration, proliferation, differentiation and morphogenesis (Niimori-Kita et al., 2014).

The other RNA binding protein, highly similar to heterogeneous nuclear ribonucleoprotein A/B (HNRPAB) transcript variant 1, was increased in SHS-exposed workers. Up-regulation of a variant of HNRPAB, was already observed in exfoliated sputum epithelial cells of workers highly exposed to environmental insults, including tobacco smoke. Monitoring HNRPAB resulted in the detection of approximately 33% more early lung cancer cases, than did the combination of X-ray and cytology (Qiao et al., 1997). Therefore, this protein might be a good initial screening test for lung carcinogenesis. Together with the previous ribosomal proteins, all three molecules are potential markers of short-term tobacco smoke exposure.

Moesin, other protein identified in this study, can elicit hyper-permeability and severe inflammatory responses (Lee et al., 2015). This protein was decreased in the nasal epithelia of SHS-exposed workers. This might result from a higher tolerance to SHS or an imbalance, after chronicle exposure. In fact, a study revealed that chronic exposure to tobacco decreased T-cell proliferation and antibody T-dependent response. In the same experiment nicotine is remarked as one of the contributors of the immunosuppressive properties of cigarette smoke (Kalra, Singh, Savage, Finch, & Sopori, 2000). Similarly, these venues workers exposed to SHS experienced a decrease in Lymphocyte antigen 6D. At earliest stage of lymphocyte differentiation, between B- and T-cell development, this protein may act as a specification marker and hence, highlight the earliest stage of B-cell specification (Consortium, 2015).

Altogether these results reliably pointed out a number of nasal epithelial proteins whose significant differences are most likely linked with SHS exposure. Therefore they should be considered valid SHS biomarker candidates and further investigated.

Conclusion

In the present study we identified 21 nasal epithelial proteins, significantly different in SHS exposed workers. Altogether, they were proved to be involved in the pathogenesis of various diseases related with tobacco smoke, either by active smoking or SHS exposure. Therefore, although these workers are clinically healthy and never smoked, at a proteomic level, they might be developing molecular mechanisms of tobacco smoke-induced diseases.

The panel of candidate biomarkers here presented, should be further validated for future application in routine screening, to flag potential progress of unnoticed diseases related with SHS exposure.

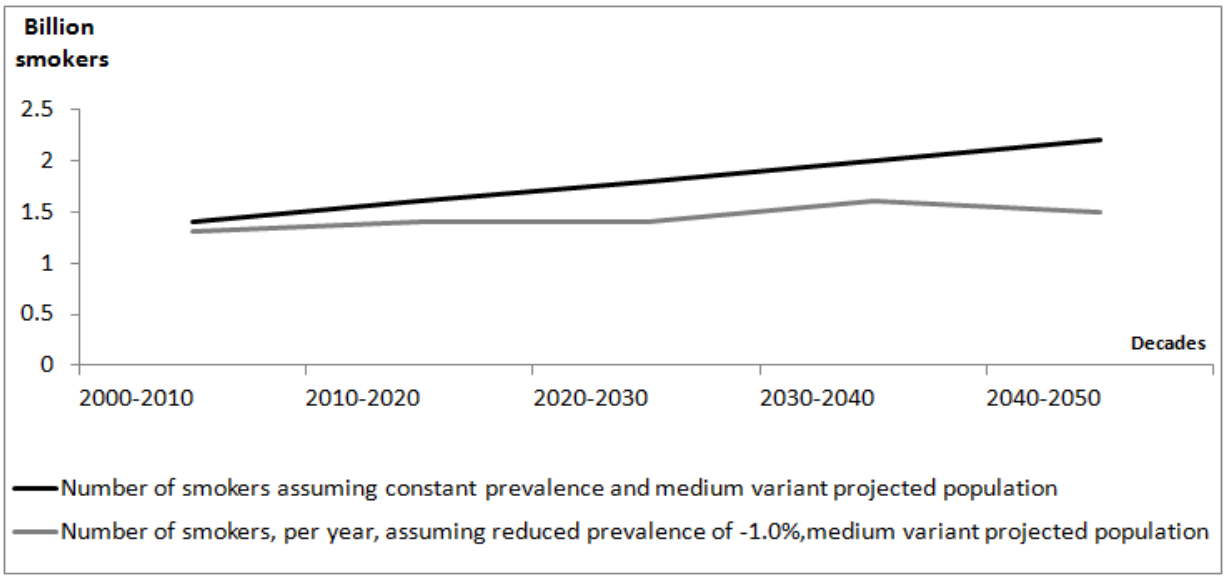
Finally this study reveals that workers from venues where it is still allowed to smoke are in danger, regardless of the protective measures adopted after the 37/2007 tobacco law.

Concluding remarks

Smoking kills many people through cancer, stroke, heart or lung disease and is the foremost preventable cause of premature death in Europe and in the United States. About ten million cigarettes are sold every minute and every eight seconds someone dies from tobacco use. Every day, nearly 100 thousand children start smoking their first cigarette, mostly appealed by their smoker relatives and advertising campaigns (WHO, 2012). It is interesting that current legislation in most countries, including Portugal, does not forbidden a minor to smoke cigarettes. Simply, they cannot purchase them (Law 37, 2007). Nonetheless, teenagers find it easy, or very easy, to have access to tobacco (DGS, 2014a).

Despite the public awareness of tobacco burden, global trends indicate that the smoking prevalence will persist, or worst, increase (Figure 20).

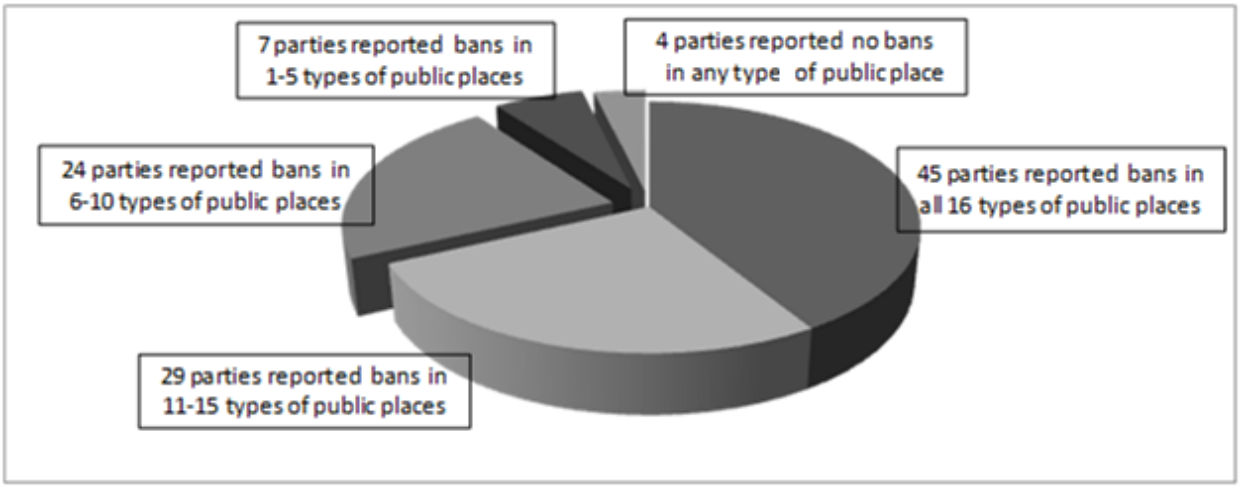
Figure 20: World smoking prevalence trend until 2050 (adapted from WHO, 2002)**.**



In 2005, the will to control the global tobacco epidemic motivated more than 170 Parties worldwide to embrace the WHO Framework Convention on Tobacco Control (FCTC). This initiate reaffirms the right of all people to the highest standard of health, by promoting and assisting countries to implement health-protective measures, without

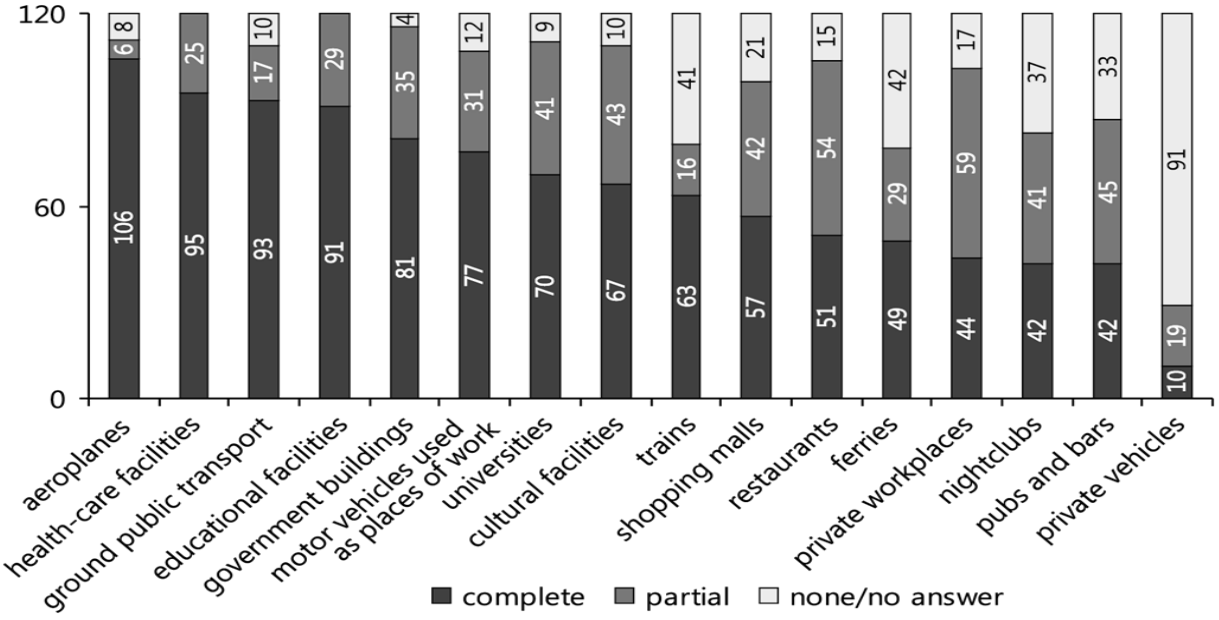
compromising their legal or constitutional rights. Total smoking bans in public places (Figure 21) are among the FCTC measures with a higher level of achievement, towards the health protection of non-smokers and also declines tobacco consumption.

Figure 21: Number of Parties, among 109, that reported to have reached complete bans on tobacco smoking five years after their commitment to FCTC, (adapted from WHO, 2012b)**.**



The workplace is a major concern, due to the long periods of SHS exposure during the working shifts. FCTC advocate for labour legislation that protects the health of the workers, whether this is in public or private buildings, restaurants or public transports (Figure 22).

Figure 22: Various degrees of bans on tobacco smoking in all 16 types of public places, among 120 parties under the FCTC (as is in WHO, 2012b).



Accordingly, in Portugal, the existing tobacco law was revised and a new law was implemented in 2008, as an attempt to increase health protective-measures. The results from the pioneer work here presented proved this assumption was not enough. Portuguese workers, from smoking-permitted venues, are not protected from SHS exposure.

The first scientific evidence resulted from the air quality assessment, where 25 Lisbon venues were monitored. Tobacco smoke is a significant source of fine respirable particulate ($PM_{2.5}$), one of the main pollutants in indoor microenvironments. The results demonstrated a significant indoor contamination of $PM_{2.5}$ in all studied restaurants where smoking is allowed. Compared with canteens or outdoor, the $PM_{2.5}$ contamination was eight times higher in smoking areas and three times higher in nearby non-smoking areas. The later finding suggests that non-smoking areas are also contaminated with tobacco smoke. Moreover, in these smoking areas the estimated levels of particulate associated polyaromatic hydrocarbons, such as the carcinogen Benzo[a]pyrene, could exceed, in one single day, more than 100 times the WHO lifetime limit, or the national legal limit (Law 102, 2010). Therefore these employees are expected to be at higher risk of developing cancer, as well as other Benzo[a]pyrene associated disorders, including immunodeficiency, respiratory and nervous system disorders.

The second piece of evidence emerged from the evaluation of effective SHS exposure. Cotinine, a metabolite of nicotine, was monitored in the urine of 97 recruited employees and was used has a biomarker of tobacco smoke inhalation. So far, cotinine is the biomarker with the highest sensitivity and specificity for tobacco combustion, retrieving accurate measurements even at low concentrations (CDC, 2005; US EPA, 1992). Cotinine is suitable for both smoking and SHS exposure assessment (Benowitz, 1996; Husgafvel-Pursiainen, 2002; Jaakkola & Jaakkola, 1997). In order to refine the results, all urine samples were collected at the end of the working shift, in the exact same day of the indoor air assessment. Additionally, the cotinine results were compared with self-reported SHS exposure obtained in a detailed survey. Even though expensive and time-consuming, surveys are foremost important, especially in studies of long-latency period for disease outcomes. Moreover, these surveys provided critical

information on specific aspects, that could affect the subjects exposure to SHS. Non-smoker employees working in smoking-designated rooms proved to be significantly exposed to higher SHS levels. In fact, they recorded over a five times increase in their cotinine levels, compared to those working in non-smoking designated areas. The proportion of smokers in those rooms, was found significantly positively correlated with cotinine and indoor $PM_{2.5}$ levels, confirming that both markers were derived from occupational SHS. The use of reinforced ventilation systems, open doors and windows, among other measures, did not prevent SHS exposure. These findings demonstrate that the partial smoking restrictions in Portuguese venues, do not bring enough improvement to indoor air quality in smoking areas and provide no protection from SHS exposure.

The subsequent studies were focused on the subclinical effects of occupational SHS exposure in never-smoker workers, to uncover possible tobacco-induced pathogenesis mechanism, unnoticed by current diagnostic tools. The choice for never-smokers avoided the bias effect of smoking in the study results. Also, the minor contribution of other sources of SHS, besides the workplace, were confirmed by the survey data.

The levels of oxidative stress parameters, including DNA breaks in blood leukocytes, the total antioxidant status and 8-OHdG, revealed no significant differences. However, after challenging blood cells with a mutagenic agent, there was a significantly lower level of DNA breaks in SHS-exposed employees, as compared to non-exposed workers. This response resembles the so called adaptive response in radiobiology, where exposure to very low doses of radiation, could induce mechanisms whereby cells become more competent to cope with subsequent exposures to high doses (Wolff, 1998). Therefore, individuals chronically exposed to SHS might possess an activated stress-induced responsive mechanism to counteracts the negative effects of tobacco smoke contaminants, which explain their prone repair response to acute DNA damage. Further studies are needed to evaluated the positive or negative consequences of this phenomena at long term.

Additional investigation was performed at protein level. The blood plasma and nasal epithelia were the samples studied to seek for candidate biomarkers by using different proteomic techniques. Blood plasma collection requires minimally invasive

procedure (venipuncture) and additionally it reaches most body elements, reflecting the changes at deepest cellular level. Nonetheless, the odds to find preliminary SHS-induced changes, are expected to increase in tissues that constantly serve as interface with the contaminated environment, and hence, the choice for a complementary approach in the nasal epithelium.

Among the plasma proteins that exhibited alteration of their abundance, CP and ITIH4 suffered the highest variation induced by SHS. Importantly, the expression patterns suggest that SHS might induce, on these acute-phase inflammation proteins, a specific proteolytic cleavage or an increased instability, possibly due to oxidative modifications. Interestingly, fragmentation patterns were already associated with a health dysfunction in the literature (Kim et al. 2011; Tewari et al. 2011; Abdullah-Soheimi et al. 2010; Squitti et al. 2008). Furthermore, in this work both acute-phase inflammation proteins were reported for the first time to be presented by a high number of isoforms. The exact pattern of fragmentation induced by SHS remains to be investigated but CP and ITIH4, might constitute biomarkers of the effects that SHS exerts on exposed workers of the hospitality industry.

Gelsolin, identified both in the pooled and individual plasma study, together with ORM2, were also relevant. Interestingly, ORM2 is another acute phase inflammation reaction protein. Both GSN and ORM2 levels were confirmed to be significantly influenced by SHS after SRM validation. These proteins are reported in numerous cancer types and stages and may be relevant for future studies. In fact, so far only a general carcinogenic principle is accepted by research community, whereby most SHS constituents need to bind DNA strains, in order to become carcinogenic. If repair mechanisms fail, permanent DNA adducts can induce cell apoptosis or lead to mutations. If mutation involves oncogenes or suppressor genes, the cancer process starts (S. S. Hecht, 2004). The complexity of the chemical mixture of tobacco makes it impossible to draw a single line between exposure and cancer (Gorini G, Gasparrini A, Fondelli MC, & Invernizzi G, 2005; Jakovljević et al., 2015). Therefore, these proteins might be key elements to uncover other carcinogenic mechanisms, which makes them critical targets in future tobacco smoke-induced carcinogenesis study. Both GSN and ORM2 are also reported in other processes associated with tobacco-related diseases,

including chronic obstructive pulmonary disease (COPD), Pulmonary Langerhans cell histiocytosis and myocardial infarction.

The nasal epithelial study also revealed a number of proteins, including EF2, RS8, RPL13 and LY6D, which significantly differentiated workers from smoking-designated areas. Again, most of these proteins abundance was found to be altered in tobacco smoke-related disease, such as COPD and cancer. Further detailed analysis revealed their influence in multiple sclerosis, Alzheimer, ataxia, emphysema, pulmonary and cardiovascular disease. These diseases translate a molecular imbalance of processes, where these proteins interfere, including protection from oxidant-induced cytotoxicity, hyper permeability and severe inflammatory responses, cell regeneration, proliferation, differentiation and morphogenesis, transport and immunity. Additionally, EF2, RS8, RL13 levels were validated individually by accurate SRM technique, which increase the confidence in this panel of biomarkers.

Considering the fact that these workers were clinically healthy, these proteomic studies proved to be a powerful tool to detect a series of potential SHS-induced subclinical molecular changes. Most of these alterations were already associated with pathological processes induced by smoking, even though they are not smokers. Therefore, we conclude that chronic occupational exposure to SHS, resemble to some extent the effects of the act of smoking, in these restaurant non-smoker workers. Altogether, the proteins indentified in this work are promising candidate biomarkers to monitor occupational SHS exposure and hence, for public health prevention.

Future perspectives

SHS is considered the major indoor air pollutant, and so far, there is no preferred method that matches the criteria of a gold standard to monitor this exposure. Ideally, a gold standard should be representative, easily and accurately measured, at an affordable cost and should be able to detect health risk, before the manifestation of the health outcome (NCR, 1986).

Most markers currently used for SHS exposure assessment are derived from studies in active-smokers (Gorini G. et al., 2005). Therefore they underestimate both the SHS exposure and the consequent health effects.

Many inherited features of SHS make it difficult to accurately monitor the exposure to this pollutant. There are multiple sources of SHS, it is heavily widespread and there is no indoor-air contaminant that is specifically related to SHS. To complicate even more, most tobacco-related diseases have a long latency period and smoking behaviors and other factors contributing to SHS exposure, have changed, especially in the last decades (NCI, 1997). Also, most tobacco smoke effects can result from acute and/or chronic exposure to SHS.

Without proper SHS exposure assessment, the estimation of the consequent health effects is compromised or limited. Indeed several exposure assessment studies have been performed, but all focus specific sub-populations. Therefore, one cannot disregard possible health outcomes that are specific to the study population, and hence, invalidate further generalization.

To overcome these limitations it is important to decrease the effect of non-SHS sources and include both present and past patterns of SHS exposure. Additionally one has to consider the type of exposure assessment. While public health studies focus SHS exposure during a certain time span, health effect studies focus on related health outcomes. Considering that all SHS exposure markers used so far have specific features, it is recommended to use a combination of indicators that cover every relevant aspect of the SHS exposure, in order to bridge the existing gaps in both study fields.

In the present work we developed a strategy in order to cover most of the SHS exposure assessment in these restaurant workers, including a detailed survey focused in SHS sources, supported by cotinine measurement; as well as indoor air monitor of contaminants, highly correlated with tobacco smoke, such as $PM_{2.5}$ and PPAH. Additionally we applied proteomic studies, as an attempt to find biomarkers sufficiently validated, to flag specific effects related with chronic exposure to SHS. Indeed the study of proteins and peptides is a landmark in basic and applied biomedical sciences.

Understanding the epigenetic mechanisms, whereby environmental tobacco smoke exposure interacts with the proteome (and genome), may help to prevent irreversible alterations of tobacco smoke in numerous cellular processes. Nevertheless the previously mentioned difficulties to assess SHS exposure together with the high cost of equipment, the need for skilled technicians and the establishment of reliable and accurate protein biomarkers makes it an arduous task.

Although one cannot overlook most constrains, in this work we contribute with a panel of biomarkers of occupational SHS exposure for non-smoker workers. The fact that they were validated individually has relevant biomarker candidates, may imply that their combined use in a panel, will provide superior power to detect SHS exposure, cover more aspects of this exposure as well as outcome scenarios.

In order to set the connection between SHS exposure and subsequent molecular mechanisms, the next step should include the characterization and validation of these plasma and nasal epithelium candidates, as well as the posttranslational modifications that might be associated with protein fragmentation and expression patterns observed. To improve their specificity these proteomic methodologies should be technically verified in 50 to 200 samples and a number of posttranslational modifications should be mapped. Together with multivariate analysis, this approach will identify false positives and prioritize a subset of proteins to be taken into more expensive and large-scale clinical validation studies. In the end, this will hopefully result in key information about these proteins, their functional roles and the pathways where they are involved. These findings will translate in a higher knowledge of pathological mechanisms that are far from completely understood, after decades of studies, including the tobacco-induced carcinogenesis. In practice, this information will improve and support monitoring and, perhaps, early diagnosis, staging and progression of tobacco-related diseases, as well

as the development of personalized therapies. Active- and former-smokers, should also be included in future studies considering that they might be specifically susceptible to SHS adverse effects.

Generally speaking, in terms of public health, the protection from SHS has improved considerably in the EU after the Council Recommendation on Smoke-free Environments in 2009 (2009/C 296/02). Accordingly, all of its signatories parties were called to take action in order to provide effective protection against exposure to SHS in enclosed public places, workplaces and public transport, no later than November 2012. About half of the Member States have adopted or strengthened their smoke-free legislation. The strictest measures were introduced by Ireland, the United Kingdom, Greece, Hungary, Bulgaria, Malta and Spain. The adoption of comprehensive legislation In these countries led to very significant drops in SHS exposure rates within a short period of time, with neutral or positive impact at economical level. According with EU directives, the positive health effects of smoke-free legislation are immediate and are supported from the vast majority of European citizens according with surveys conducted at EU and at national level. However some Member States are lagging behind, in part due to complex legislation that include exceptions. Recently the Portuguese government adopted amendments to the current legislation, which includes the creation of smoke-free places. Nonetheless, it is only effective within five years, hence all the establishments that already have smoking areas, in accordance with the law in force (law 37/2007), will be allowed to keep them until the end of 2020. From then on, a number of exceptions will still allow smoking in the rooms and halls of shows and other places of the arts and broadcasting; at the fairgrounds, in casinos, bingo halls, game rooms and other grounds for non-artistic nature shows; in the precincts of exhibitions and fairs; in hotels and similar accommodation services; in catering or drink establishments, including those with areas to dance. Transport services will also allow smoking in railway and bus stations, in maritime and river marshalling and in airports, although this issue is not on the state level. It also keeps the possibility of smoking rooms in hotels and similar and where there can be placeholders for smoking, if they satisfy specific requirements and do not have any service, including bar and restaurant. There is no ban on smoking in outdoor spaces, including terraces (Portugal Gov., 2015 & COPPT, 2015).

From this work, it is strongly recommended the urgent implementation of legislation that effectively protects public health.

In Portugal, 30 persons dye everyday due to smoking or SHS exposure (COPPT, 2015). Action taken today will determine tomorrow's reality!

Highlights

There is no safe level of SHS exposure

No protective measures adopted after the 37/2007 law were effective

There are no validated biomarkers specific for SHS exposure assessment. Together with the chronic nature of occupational SHS-exposure, it might result in disease and death

Workers from venues where it is still allowed to smoke might be developing subclinical disease

It is urgent to ban tobacco smoke from all indoor places

This work presents a robust, efficient and suited proteomics methodology to screen candidate biomarkers in complex biological samples

A proteomic profile associated with tobacco smoke-induced diseases was indentified in a group of healthy non-smokers working in smoking-designed areas

These findings might translate the critical role of proteins involved in SHS-induced response

A panel of proteins is proposed as promising candidate biomarkers of SHS exposure
including CP, ITIH4, GSN and ORM2 in blood plasma
and EF2, RS8, RPL11 and LY6D in nasal epithelium

The panel might flag the unnoticed progress of tobacco smoke related diseases

Further investigation and validation are needed, to select the most relevant biomarker candidates, for future routine screening of occupational SHS-exposure, towards public health protection

Supplementary information

Table 16: Characteristics of the worker's population.

Qualitative data						
		N (%)	**SDA (%)**	**NSDA (%)**		
Gender	**Female**	24 (25)	9 (9)	15 (16)		
	Male	72 (75)	39 (41)	33 (34)		
Smoking status	**Current Smoker**	25 (26)	14 (15)	11 (11)		
	Non smoker*	71 (74)	34 (35)	37 (39)		
Other ETS exposure ****	**Yes**	43 (45)	26 (27)	17 (18)		
	No	53 (55)	22 (23)	31 (32)		
Quantitative data						
		Median	**Min**	**Max**	**Mean**	**(SD)**
Age	**SDA**	32	20	66	36	-11.5
	NSDA	45	18	66	43	-12.5
Nº of cigars (by day)***	**SDA**	20	7	30	19.5	-7
	NSDA	10	3	20	11.9	-5.9
Years of smoking habit***	**SDA**	13.5	5	26	15.1	-7.2
	NSDA	29.5	3	49	26.2	-12.8
Months in the current job	**SDA**	36	1	528	81.4	-108.5
	NSDA	96	1	468	123.9	-133.1
Hour in a week of service	**SDA**	40	14	90	41	-11.6
	NSDA	48	14	66	47.8	-11.3

* Never smoked, or did not smoke for at least 6 months prior to sample collection;** Exposure outside the workplace in the study, e.g. at home, transports, second job, etc);*** In the current smoker workers

Table 17: Comparison of fine particulate matter ($PM_{2.5}$), Particulate polycyclic aromatic hydrocarbons (PPAH) and carbon monoxide (CO) levels studied per restaurant, based on a statistical Mann Whitney U test for each pair of sites (Sro, NSro, SFre) with Holms sequential Bonferroni adjustment of the significance level (adjusted α).

Parameters	Sites compared	p value	adjusted α	Mann-Whitney U
PM2.5 or PPAH	Sro vs SFre	0*	0.017	0
	Sro vs NSro	0*	0.025	0
	NSro vs SFre	0.96	0.05	77
CO	Sro vs SFre	0.02*	0.017	39
	Sro vs NSro	0.09	0.025	47
	NSro vs SFre	0.3	0.05	59

Sro, Smoking rooms; Nsro, Non-Smoking rooms; SFre, Smoke-Free restaurants.

Table 18: Work and household details collected in the interview.

		NSNE		NSE		SNE		SE		Total
		Nº	%*	Nº	% *	Nº	%*	Nº	%*	Nº
		38		32		12		14		96
Do you have another job? Name the correspondent category.										
No		27	96	21	84	10	100	5	83	63
Yes:		1	4	4	16			1	17	6
	Technical			1	4					1
	Fabricators and labourers	1	4	1	4			1	17	3
	Education and sports			1	4					1
	Hospitality			1	4					1
	Nº of workers who answered	28		25		10		6		69
Did you have other jobs before? Name the correspondent category.										
Yes:	Sales and administrative support	3	11	2	8	1	10			6
	Service		0	1	4	2	20			3
	Fabricators and labourers	8	29	3	12			2	33	13
	Farming	3	11	1	4					4
	Education and sports		0	3	12					3
	Hospitality	14	50	15	60	7	70	4	67	40
	Nº of workers who answered	28		25		10		6		69
Are you exposed to SHS at home?										
No		22	79	19	79	6	60	5	83	52
Yes:		6	21	5	21	4	40	1	17	16
	Nº of workers who answered	28		24		10		6		68
In case you do, please give an estimate of how many hours you're SHS exposed at home.										
1h		3	60	4	80	1	33	1	100	9
2h		2	40	1	20	2	67			5
	Nº of workers who answered	5		5		3		1		14
Nº of smokers at home?										
1		4	67	5	100	3	75	1	100	13
2		1	17			1	25			2
7		1	17							1
	Nº of workers who answered	6		5		4		1		16
Nº of cigarettes your cohabitants smoke at home?										
1		3	60	4	80	1	33.3	1	100	9
2		2	40	1	20	1	33.3			4
3						1	33.3			1
	Nº of workers who answered	5		5		3		1		14
Do you have a fireplace at home? If so, how many times in a week do you light the fire?										
No		22	79	19	76	10	100	6	100	57
Yes:		6	21	6	24					12
Warm season	**<3**									
	≥3	1	17	4	67					5
Cold season	**<3**	3	50	2	33					5
	≥3	2	33							2
	Nº of workers who answered	28		25		10		6		69

*The percentages include only the workers who answered.

Table 19: Lifestyle details, including diet and sports activity, collected in the interview.

		NSNE		NSE		SNE		SE		Total
		Nº	%*	Nº	%*	Nº	%*	Nº	%*	N.º
		38		32		12		14		96
Do you drink coffee or tea? In case you do, how many coffees or cups of tea do you drink in a day?										
No		8	29	6	24	3	30	0	0	17
Yes:	**≤3**	19	68	14	56	3	30	3	50	39
	>3	1	4	5	20	4	40	3	50	13
Nº of workers who answered		28		25		10		6		69
Do you drink alcoholic beverages? How many cups/units in a day?										
No		4	11	4	12	2	17	2	14	12
Yes:	**≤2**	2	5	3	9	0	0	6	43	11
	3-4	27	71	20	61	7	58	4	29	58
	≥5	5	13	6	18	3	25	2	14	16
Nº of workers who answered		38		33		12		14		97
What type of alcoholic beverages do you usually drink?										
Beer and/or Wine		4	12	2	7	0	0	5	42	11
Beer/Wine/Other(s)		30	88	27	93	10	100	7	58	74
Nº of workers who answered		34		29		10		12		85
How many meals you do in a day?										
x		4	14	6	25	2	20	1	17	13
3		14	50	10	42	5	50	3	50	32
≥4		10	36	8	33	3	30	2	33	23
Nº of workers who answered		28		24		10		6		68
From the following, list which diet do you follow?										
Vegetarian				1	4					1
Specific diet		1	3	2	7					3
Equal amounts of Meat and fish		28	74	21	78	10	83	5	71	64
More fish than meat		9	24	3	11	2	17	2	29	16
Nº of workers who answered		38		27		12		7		84
Do you practice any sports activity? In case you do, how often in a week?										
No		17	61	15	65	9	90	5	83	46
Yes:	**<3**	8	29	3	13	0	0	0	0	11
	≥3	3	11	5	22	1	10	1	17	10
Nº of workers who answered		28		23		10		6		67

*The percentages include only the workers who answered.

Table 20: SRM assay targeting GSN and ORM2 peptides.

Protein Accession nº (Gene Name)	Peptide sequence	Position in the sequence*	Collision Energy	Retention time	Transitions	
					Precursor Mz	Product Mz
P19652 (ORM2)	WFYIASAFR	52-60	20.3	63.2	580.80	827.44
						664.38
						551.29
						480.26
	TEDTIFLR	74-81	17.8	36.2	497.76	764.43
						649.40
						435.27
P06396 (GSN)	EPGLQIWR	65-72	17.9	42.2	499.77	772.45
						602.34
						474.28
	EVQGFESATFLGYFK	148-162	28.8	67.3	861.92	1162.58
						1033.54
						747.87
	HVVPNEVVVQR	178-188	22.1	23.8	638.36	1138.66
						1039.59
						940.52
	SEDCFILDHGK	328-338	22.7	29.3	660.80	682.39
						569.30
						552.76
	LFACSNK	669-675	15.5	19.1	420.21	726.32
						579.26
						508.22

*Peptide position in the protein sequence: the convention for writing the amino acid sequence of a protein is to start by the primary translated N-terminal tail, on the left side, until the C-terminal end, on the right side of the sequence (Twyman, 2013).

Table 21: SRM assay for RS8, EF2, RL13 and LY6D, with the targeted peptide sequence, position, collision energy (CE), retention time (RT) and the transition duo with the precursor and product ion mass-to-charge ratio (Mz).

Gene Name (Length)	Sequence	Position*	CE	RT	Transitions	
					Precursor Mz	Product Mz
RS8 (208)	IIDVVYNASNNELVR	77-91	28.7	47.53	859.957	1179.575
						1016.512
						831.432
	NCIVLIDSTPYR	98-109	24.7	43.01	725.872	964.510
						738.342
						435.235
	ISSLLEEQFQQGK	157-169	25.5	39.71	753.894	993.464
						864.421
						607.320
EF2 (858)	TFCQLILDPIFK	287-298	25.3	64.3	747.905	1086.656
						958.597
						845.513
						732.429
						619.345
						504.318
						623.847
	ETVSEESNVLCLSK	580-593	26.8	36.26	797.885	1265.604
						1049.530
						920.487
						833.455
						719.412
						620.344
						507.260
RL13 (211)	GFSLEELR	74-81	17.2	36.19	475.7507	746.404
						659.372
						546.288
						417.246
	STESLQANVQR	105-115	21.4	15.72	616.8151	915.501
						828.469
						715.385
						587.326
						516.289
						402.246
						522.775
	LATQLTGPVMPVR	145-157	23.7	41.3	691.8949	1097.614
						969.555
						856.471
						755.423
						698.402
						502.281
						599.834
LY6D (128)	TTNTVEPLR	46-54	18.4	20.94	515.78	828.457
						714.415
						613.367
						414.732

*Peptide position in the protein sequence: the convention for writing the amino acid sequence of a protein is to start by the primary translated N-terminal tail, on the left side, until the C-terminal end, on the right side of the sequence (Twyman, 2013).

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